University of Alberta

EVALUATION OF THE EFFECTIVENESS OF SERTOLI CELLS TO SUPPRESS IMMUNE RESPONSES FOR THE POTENTIAL USE AS A SAFER ALTERNATIVE TO SYSTEMIC IMMUNE SUPPRESSION IN CLINICAL ISLET TRANSPLANTATION FOR THE TREATMENT OF TYPE 1 DIABETES

by

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This text is dedicated to my sister Hannah and all those affected by diabetes. May you some day know a life free from insulin and the ravages of diabetes.

ABSTRACT

Islet transplantation using what has come to be known as the Edmonton protocol has been tremendously successful at helping dramatically improve blood glucose control and quality of life in many patients with type 1 diabetes. However, risks associated with long-term immunosuppressive therapy and a shortage of donor organs for transplantation currently limit this procedure to patients with brittle type 1 diabetes. One alternative to long-term immune suppression that has been studied experimentally is the co-transplantation of islets with Sertoli cells.

Sertoli cells have been called the "nurse cells" of the testes as they aid in the growth and development of new sperm as well as protect sperm from harmful immune destruction. Many labs have successfully re-created the immune privileged environment of the testis beneath the kidney capsule of diabetic rodents where optimal numbers of Sertoli cells have been shown to protect islet allo-, and xenografts, as well as islet isografts, in non-obese diabetic mice, from immune destruction. However, too few or too many Sertoli cells co-transplanted with islets led to a decrease in islet graft survival time. Furthermore, the mechanism of how Sertoli cells protect islet allografts from immune destruction is still not fully understood.

This dissertation focused on optimizing islet/Sertoli cell co-transplantation conditions in an allogeneic mouse model of chemically induced diabetes. We additionally examined the effect of factors secreted by Sertoli cells on immune effector mechanisms.

Co-transplantation of islets with Sertoli cells was found to have no deleterious effect on islet graft function in a syngeneic mouse model of chemically induced diabetes. Furthermore, Sertoli cells were able to extend islet allograft survival under certain conditions. We observed a correlation between extended islet allograft survival, upon co-transplantation with Sertoli cells, and Sertoli cell tubule formation post-transplantation. Graft success and potentially tubule formation were also found to be dependent on Sertoli cell number as well as recipient mouse strain. Furthermore, we found that Sertoli cells could effectively protect islet allografts from CD8 T-cell infiltrate but not CD4 T-cell and macrophage infiltrate.

Further examination of the effect of factors secreted by Sertoli cells on the cytotoxic T lymphocyte granzyme B pathway to cell death, a main effector mechanism in graft destruction, showed that a factor secreted by Sertoli cells effectively inhibited granzyme B enzymatic and biological (killing) activity through the formation of a stable complex. We suspected that this factor was serine proteinase inhibitor-6, a potent inhibitor of granzyme B known to form an essentially irreversible complex with granzyme B. However, staining for serine proteinase inhibitor-6 in Western blots showed that it was not the factor from Sertoli cells in the granzyme B complex. This led us to believe that this factor from Sertoli cells may be some other, currently unidentified, serpin. Our lab is currently working on identifying this factor using mass spectroscopic analysis.

These experiments provide important information on how to optimize islet/Sertoli cell co-transplantation conditions and identify one mechanism of how Sertoli cells protect themselves and bystander cells from immune destruction.

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LIST OF ABBREVIATIONS

α	alpha
ADP	adenosine diphosphate
Adv	adenovirus
ANOVA	analysis of variance
APCs	antigen presenting cells
ATP	adenosine triphosphate
B6	C57BL/6
β	beta
BSA	bovine serum albumin
C3H	C3H-HeJ
C57	C57BL/6 CTL cell line
CD	cluster of differentiation
CD-MPR	cation dependent-mannose-6 phosphate receptor
CI-MPR	cation independent-mannose-6 phosphate receptor
Con-A	concanavalin-A
CTLA-4	cytotoxic T lymphocyte antigen-4
CTLs	cytotoxic T lymphocytes
δ	delta
DAB	diaminobenzadine
DC	dendritic cell
DCCT	Diabetes Control and Complications Trial
Degran grB	mouse degranulate granzyme B

DENIS	Deutsche Nicotinamide Intervention Study
DMEM	Dulbecco's modified Eagle's medium
DPT-1	Diabetes Prevention Trial-Type 1
EGTA	ethylene glycol tetra acetic acid
EMEM	Earl's minimum essential medium
ENDIT	European Nicotinamide Diabetes Intervention Trial
ES	embryonic stem
FACS	fluorescence activated cell sorter
FAD	flavin adenine dinucleotide
FasL	Fas ligand
FBS	fetal bovine serum
FITC	Fluorescein Isothiocyanate
FSH	follicle stimulating hormone
Galα(1,3)Gal	galactose α(1,3)-galactose
GIP	glucose-dependent insulinotropic polypeptide
GLUT2	glucose transporter 2
grB	granzyme B
H/E	Hematoxylin/Eosin
HBSS	Hanks' balanced salt solution
HLA	human leukocyte antigen
hp grB	human purified granzyme B
IEPD	isoleucine/glutamate/proline/aspartate
IFN-γ	interferon-gamma

Ig	immunoglobulin
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IL-1	interleukin-1
M6P	mannose 6-phosphorylated
M6P/IGF-II	mannose-6 phosphate/insulin-like growth factor-II
MFI	mean fluorescence intensity
MHC-II	major histocompatibility complex class-II
MPR	mannose-6 phosphate receptor
NOD	non-obese diabetic
PBS	phosphate buffered saline
PERV	porcine endogenous retrovirus
PI-9	proteinase inhibitor-9
pNA	paranitroanalide
pp	polypeptide
P/S	penicillin/streptomycin
SCCM	Sertoli cell conditioned media
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
S1	signal 1
S2	signal 2
SPI-6	serine proteinase inhibitor-6
TBS-T	Tris buffered saline with Tween-20

T-cell	thymocyte-cell
TCR	T cell receptor
TGF-α	transforming growth factor-alpha
TGF-β	transforming growth factor-beta
Th1	T helper cell 1
Th2	T helper cell 2
TNF-α	tumor necrosis factor-alpha
TRIGR	Trial to Reduce Type 1 Diabetes in the Genetically at Risk
TUNEL	TdT-mediated dUTP nick end labeling

CHAPTER I

GENERAL INTRODUCTION

I-A) DIABETES MELLITUS

Diabetes is a serious health concern in Canada for many reasons. It is estimated that diabetes affects over 2 million Canadians with 60,000 new cases each year. Diabetes is a major cause of coronary artery disease, which is a leading cause of death in Canada. It is also a leading cause of new cases of adult blindness and kidney disease. Diabetic neuropathy can also be very debilitating for people with diabetes leading to such complications as amputation and erectile dysfunction. In all, these complications end up disabling people with diabetes at middle age, ultimately resulting in a shortened life span. The economic burden of diabetes and its complications in Canada is estimated to be up to $9 billion annually in direct and indirect health care costs. It is clear that the eradication of diabetes and/or related complications needs to be a major focus of research in Canada (1).

Diabetes Mellitus is a metabolic disorder characterized by hyperglycemia due to impaired insulin secretion, action or both (2). There are three main types of diabetes: type 1, type 2, and gestational diabetes (3). Type 2 is the most common form of diabetes as it affects approximately 90% of people with diabetes. People with type 2 diabetes can have a combination of problems with insulin secretion as well as action. It is more prevalent in people over the age of 40 and is strongly linked to obesity and physical inactivity; however, genetics and ethnic background are also thought to play a role in its

development. Woman diagnosed with gestational diabetes, characterized by hyperglycemia during pregnancy, are at increased risk of developing type 2 diabetes. In people with type 2 diabetes, blood glucose is managed with diet, exercise, medications and sometimes insulin. The good news about type 2 diabetes and its complications is that with proper lifestyle modifications it may be prevented (4-9). Unfortunately for people with type 1 diabetes, often diagnosed during childhood, there is no known way to prevent it, resulting in a lifetime dependence on injected insulin for survival and an increased risk of late complications (10).

1. Type 1 Diabetes

Type 1, formerly known as juvenile or insulin-dependent, diabetes affects approximately 10% of people with diabetes. Type 1 diabetes results from the autoimmune destruction of the insulin secreting beta (β)-cells located in the islets of Langerhans of the pancreas (11). Destruction of the β-cells results in insulin deficiency and an absolute requirement for exogenous insulin administration for survival. Before the discovery of insulin in the early 1920's, type 1 diabetes was managed with a diet low in carbohydrates which led to patients eventually succumbing to diabetic ketoacidosis (12). Diagnosis with type 1 diabetes is no longer a death sentence but with the introduction of insulin has been promoted to a chronic, incurable illness with many debilitating and life threatening acute and chronic complications such as hypoglycemia, diabetic ketoacidosis, atherosclerosis, heart disease, retinopathy, neuropathy, and nephropathy (10). Currently there is no known way of preventing type 1 diabetes; however, treatments aimed at restoring glycemic control may delay or prevent the complications of this disease (13).

a) Etiology of type 1 diabetes and disease pathogenesis

In type 1 diabetes an environmental trigger(s) is thought to initiate an autoimmune response against pancreatic β-cells in those individuals who are genetically at risk (11;14). The progressive loss of β-cell mass over time results in pre-diabetic glucose intolerance and eventually symptoms of diabetes such as increased thirst and urine output and, in severe cases, diabetic ketoacidosis eventually leading to diabetic coma and, if untreated, death (15). Both humoral and cell-mediated immunity are involved in the progressive destruction of β-cells that leads to type 1 diabetes. One model suggests that some sort of environmental trigger, a virus for example, causes β-cell destruction and the release of proteins that are taken up by resident antigen presenting cells (APCs; macrophages, monocytes, and dendritic cells) and presented in the context of major histocompatibility complex class II (MHC-II) molecules to lymphocytes (16). Production of interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) by macrophages and lymphocytes leads to the induction of free radicals in the β-cell which modify β-cell proteins, making them more immunogenic, aggravating the immune system even further. Cytotoxic T lymphocytes (CTLs) also destroy β-cells through the Fas/Fas ligand (FasL) and granzyme B pathways and B cells release autoantibodies to islet antigens (islet cell cytoplasm, insulin, glutamic acid decarboxylase, and protein tyrosine phosphatase) (16). The interplay between genetic susceptibility and environmental trigger(s) is not fully understood but several theories exist as to how this aggravated immune response to β-cells is initiated.

i) genetic factors

The general population risk of developing type 1 diabetes is approximately 1 in 300. This proportion is magnified 12 to 20 fold in first-degree relatives of people with type 1 diabetes. The most important genes that provide both susceptibility towards, and protection from, type 1 diabetes are located within the MHC-II region on chromosome 6p21 (17). Genes at this loci account for 45% of genetic susceptibility to type 1 diabetes. MHC-II molecules are expressed primarily on APCs (macrophages, dendritic cells, and B cells), where they present processed antigenic peptides to immune cells resulting in the initiation of humoral and cell-mediated immune responses (18). For this reason it makes sense that certain inherited MHC molecules could increase disease susceptibility and trigger immune responses while others confer disease protection. However, it is still unclear at this time how MHC-II molecules play a role in the disease pathogenesis of type 1 diabetes. Certain haplotypes (human leukocyte antigen [HLA]-DR3 and HLA-DR4) are associated with an increased risk for diabetes, others (HLA-DR2) are associated with strong protection from the disease, with protection being dominant over susceptibility (19). There are also a number of non-HLA loci associated with susceptibility to type 1 diabetes now identified (20). However, the function of genes at only two of these non-HLA loci are known. IDDM2 on chromosome 11p5.5 is a polymorphic region that maps to a variable number of tandem mini-satellite repeats and IDDM12 on chromosome 2q33, which has an apparent action on the CTL-associated protein 4 that is responsible for modulating immune responsiveness (21;22). It is possible to test for genetic susceptibility to type 1 diabetes but testing of this kind is not current practice due mainly to the absence of an appropriate means to prevent the onset

and progression of the disease. Furthermore, having genetic susceptibility does not automatically mean the individual will develop diabetes. A low concordance rate (35-50%) of type 1 diabetes among monozygotic twins clearly indicates that genetic risk factors may be necessary but are not sufficient for the disease to occur (23). For this reason any form of primary prevention of type 1 diabetes based on genotyping alone must be completely benign so as to not harm healthy individuals who would potentially not even develop the disease. These types of interventions will most likely come from a better understanding of environmental disease triggers and the subsequent progression of disease pathogenesis.

ii) environmental factors

The traditional view of type 1 diabetes postulates that an environmental agent triggers disease onset in genetically susceptible individuals. However, the interplay between environment and genetics may be more complex than originally postulated with the onset of heritable immune dysregulation coming about due to a life-long influence of multiple environmental factors such as infectious agents, dietary factors, and environmental toxins as well as new classes of influencing variables (sanitation, health care access, and vaccinations) (15).

To date 15 different viruses have been reported to be associated with the development of type 1 diabetes in human and animal models. These include coxsackie B virus, rubella virus, mumps virus, cytomegalovirus, Epstein-Barr virus, and Varicella Zostar virus in humans and nine other viruses have been reported to be associated with the development of type 1 diabetes in animals including mice, rats, hamsters, cattle, pigs, and non-human primates (24). One observation that led to the thinking that viruses may

be involved in the onset of type 1 diabetes was the apparent seasonal incidence in the onset of acute disease with a peak in autumn (25). Diseases with seasonal incidences are often caused by viral infections. Other reports have indicated a viral infection at the time of or just before the onset of type 1 diabetes with virus being isolated from the pancreas of deceased diabetic patients (26). In addition, it is possible to experimentally infect animals with viruses to induce diabetes and a virus-induced, diabetic, transgenic mouse has been created (27-29). The mechanism of how viruses induce autoimmunity to β-cells has been examined and several theories exist as to how the disease may be triggered. Infectious agents such as viruses and bacteria have been shown to trigger immune responses to β-cells via the release of sequestered β-cell antigens and/or by mimicking these antigens (30). Normally any T cells that have a high affinity for self-MHC or self-MHC and self-antigen are destroyed during negative selection in the thymus (31). However, certain tissue specific antigens (for example those found in the pancreatic β-cells) may be sequestered and not expressed in the thymus resulting in the release of auto-reactive T cells into the periphery. However, once in the periphery these auto-reactive T cells are unable to mount an immune response upon encounter with antigen in the absence of co-stimulation. Viral and bacterial infections may either release sequestered antigen and/or result in the expression of co-stimulatory molecule on the APC resulting in an inappropriate immune response to self antigen (30). Viruses and bacteria have also been shown to express antigenic determinants that are identical or similar to normal host-cell components. In this case when the host mounts an immune response to the pathogen, antibodies generated to clear the pathogen can cross react with healthy host tissue and cause an immune response to host tissue (30). A third possibility

is that virus directly infects the pancreatic β-cells causing them to die via apoptosis. Apoptotic bodies from the β-cell would be taken up by resident macrophages in the pancreas which would in turn present the β-cell antigens to the immune system to initiate humoral and cellular-mediated immune responses. A final mechanism that has been proposed involves a breakdown in immune balance between T helper cell 1 (Th1) and T helper cell (Th2) subtypes (24). In this case Th1 and Th2 cell subtypes are presumed to be present in equal amounts until a virus results in the activation of macrophages and cluster of differentiation (CD)8 positive T cells, which in turn results in an increase in the number of Th1 cells and ultimately an inflammatory response to β-cells. The mechanism used by viruses to trigger type 1 diabetes may depend on the virus. In any event it may be possible to attenuate this immune response by treating genetically at risk individuals for type 1 diabetes with viral vaccines (32).

A second environmental trigger for type 1 diabetes may be the early introduction of cow's milk or the short duration of breast feeding in infants who are genetically at risk (33;34). Cow's milk contains five principal proteins: casein, beta-lactoglobulin, alpha-lactalbumin, gamma-globulin, and bovine serum albumin (BSA). Immunoglobulin (Ig)G antibodies to cow's milk proteins are present in almost all infants exposed to cow's milk (35). The titers of these cow's milk antibodies decrease with age, but remain high in individuals with celiac disease (35). Celiac disease is associated with HLA-DQ2 (A1*0501, B1*0201) which is present on the extended HLA haplotype A1-B8-DR3, as well as A30-B18-DR3, well known to also confer risk for type 1 diabetes (36-38). It is proposed that molecular mimicry also plays a role in the initiation of autoimmunity associated with cow's milk proteins in that antibodies to cow's milk proteins cross-react

with β-cell antigens (39). Another theory suggests that breast milk contains growth factors, cytokines, and other immunomodulatory agents that promote functional maturation of the intestinal mucosal tissues, a major immunoregulatory barrier (33). If, for example, an infant has early exposure to cow's milk before the development of the gut mucosa, proteins from cow's milk (which just happens to be the first antigen encountered) result in inappropriate immune response to self in those who are genetically susceptible . However, avoidance of cow's milk altogether is potentially not an option either due to the finding that vitamin D in milk can offer protection from autoimmune diabetes (40).

iii) prevention strategies for type 1 diabetes

Currently there is no known way of preventing type 1 diabetes, however, several clinical trials are underway in an attempt to discover how to delay or prevent the onset of this disease. The majority of clinical studies for the prevention of type 1 diabetes enroll individuals deemed to be at a high-risk for developing diabetes. For example, people with a high genetic predisposition for diabetes (first-degree relatives), and who are islet autoantibody positive. Many treatments are being tested for their ability to prevent type 1 diabetes including: nicotinamide (Deutsche Nicotinamide Intervention Study [DENIS] and the European Nicotinamide Diabetes Intervention Trial [ENDIT]), avoidance of cow's milk in infants (Trial to Reduce Type 1 Diabetes in the Genetically at Risk [TRIGR]), and insulin antigen therapy (Diabetes Prevention Trial-Type 1 [DPT-1]) (41). All of these studies are based on initial success in animal models of diabetes and all of these interventions are benign in nature as it is not possible to identify those individuals who will go on to develop type 1 diabetes with 100% certainty.

To date, none of these interventions have been shown to work well enough to be recommended to patients for the prevention of type 1 diabetes. The major advance that these trials have brought to the field of diabetes research is the ability to determine those individuals in the population who are at a high risk for developing type 1 diabetes with genetic, and early autoimmune indicators such that when metabolic symptoms of overt diabetes occur they are picked up much earlier. Early detection of diabetes may help reduce deaths at the onset.

Finally, an avenue of early intervention for the prevention of type 1 diabetes that is just beginning to be explored in humans is the development of vaccine therapies. Vaccines may work in various ways, including changing the immune response from a destructive Th1 to a more benign Th2 response, inducing antigen-specific regulatory T cells, deleting autoreactive T cells, or preventing immune cell interaction (42). Most prevention studies with vaccines have been studied in animal models rather than humans to date. The non-obese diabetic (NOD) mouse is an animal model of autoimmune type 1 diabetes that is commonly used for testing early interventions for the prevention of diabetes (43). Unfortunately, diabetes seems to be very easily prevented in the NOD mouse model and certain interventions that work to prevent diabetes in the NOD do not translate to effectiveness in preventing disease in humans. Even though many avenues are being explored and many lessons are being learned as clinical trials for the prevention of type 1 diabetes continue, there is still a need to focus research attention on understanding exactly how diabetes ensues in the first place before long term solutions can be found. In the absence of a real means of preventing type 1 diabetes the focus turns

to optimizing diabetes treatment for the prevention of complications in order to extend the quality and duration of life of people affected with this insidious disease.

b) Treatments for type 1 diabetes

Undeniably the most outstanding achievement in diabetes research was the discovery of insulin in the early 1920's by Banting, Best, Collip, and McLeod. It was known that people with diabetes could not tolerate 'sugar' and before the discovery of insulin as the active ingredient in the pancreas, responsible for blood glucose control, people with diabetes were put on a low carbohydrate, or essentially starvation diets. These early patients appeared anorexic as their bodies wasted away into nothing. The advent of insulin therapy allowed people with diabetes to tolerate a regular diet as it greatly improved their blood glucose control resulting in a longer life span (12). However, even today, with advances in insulin formulations and administration, micro- and macrovascular complications, such as neuropathy, nephropathy, retinopathy, and cardiovascular disease, are common causes of morbidity and mortality in patients with type 1 diabetes (10). This is a result of the inability of injected or infused insulin to fully restore metabolic control in these patients, with transient periods of hyperglycemia (high blood glucose levels) leading to blood vessel damage and the above mentioned complications. Only through intensive insulin regimes can near normoglycemia be approached and microvascular complications be delayed or prevented (13). However, intensive insulin therapy can result in an increased number of hypoglycemic (low blood glucose levels) episodes which can interfere with daily tasks as well as have serious health consequences. Additionally, intensive insulin therapy and management may be difficult to achieve in all patients with type 1 diabetes (44). Advances in insulin pump

technology and continuous blood glucose monitoring may make it easier to manage blood glucose (45;46). Truly what is needed is a more physiological approach to regulating blood glucose that combines the idea of continuous blood glucose sensing and appropriate insulin delivery to maintain tight glycemic control and avoid complications. Cell replacement therapy, either through pancreas or islet transplantation, has now become a clinical reality for a select few patients with diabetes offering the potential to restore glycemic control and eliminate the chronic complications associated with insulin therapy (47-53). However, both pancreas and islet transplantation are not without their challenges. Both currently rely on a limited supply of human pancreata and are accompanied by immunosuppressive therapy (53). Engineering non-beta cells to secrete insulin within the diabetic may be another possible way to treat diabetes without the need for immunosuppression (54;55). Of course with each new solution comes a new challenge but at least many avenues are being explored to improve treatment options for those affected by diabetes.

i) insulin formulations

The discovery of insulin was a huge breakthrough in the treatment of type 1 diabetes; however, insulin injection is not a cure for diabetes in that it does not fully restore metabolic control (56). Additionally, too much insulin can lead to acute complications such as hypoglycemia and potential coma and death, too little insulin can result in periods of hyperglycemia resulting in damage to blood vessels and numerous life threatening chronic complications (10). Despite the limitations of insulin therapy many advances have been made in insulin formulations that help patients keep their blood glucose under better control (57). Initially, insulin for the treatment of diabetes was

extracted from bovine and porcine pancreata. Human insulin did not become available until the early 1980's. With the development of recombinant DNA technology biosynthetically made insulin was possible and soon became the preferred method of production. This technology allowed for the engineering of insulin analogues with improved pharmacokinetic properties for subcutaneous administration (58-60). Of the 180 branded insulin preparations available today, two main groups based on their duration of action in the body can be described: prandial bolus insulin (short acting), and basal insulin (long acting) (57). Soluble insulin is typically taken as a bolus at meal time and the dose is based on the nutrient content of the meal. Basal or background insulin is taken usually in the morning and before bed to keep blood glucose levels in check between meals. Combination therapy of both short and long acting insulin formulations can be very effective in managing blood glucose control (57).

Despite the demands of a variable lifestyle with changes in daily nutritional intake, physical activity, and other physiological, and psychological demands the pancreas of a healthy individual is able to maintain tight control of plasma glucose levels (3.5-7.0 mmol/L). After eating, plasma glucose concentrations rise to a peak in 30-60 minutes and return to basal concentrations, or below, within 2-3 hours. Similarly, insulin concentrations closely rise and fall with plasma glucose concentrations. Management of type 1 diabetes should aim to closely mimic normal physiology (57). Insulin should peak at the same time blood glucose peaks and it should be rapidly cleared from the blood as glucose is cleared. Current insulin formulations approach, but do not mimic, the normal pancreatic or insulin secretory responses of individuals without diabetes. Soluble or regular insulin (short-acting) has an initial lag phase that is followed by an increase in

plasma insulin levels and returns to the basal level only after 6-8 hours (61). Due to the initial lag phase of soluble insulin, people using this formulation have to inject 30 minutes before they eat their meal. New insulin analogues such as insulin aspart and insulin lispro, so called rapid acting insulin formulations, have a much more rapid onset of action and can be taken with or after a meal (62;63). The reason soluble insulin has such a slow peak action is because the insulin monomers tend to associate with themselves. Insulin analogues are created with single amino acid substitutions to decrease the tendency of insulin monomers to associate with themselves thus increasing their solubility and absorption by the subcutaneous tissue (60). Due to these improvements fewer fluctuations in blood glucose are seen with the analogues compared with regular soluble insulin. However, rapid acting insulin alone, although an improvement from regular insulin, is not enough to achieve good glycosylated haemoglobin values. This is because of inadequate basal insulin supplementation (64). Basal insulin formulations are the opposite of soluble formulations in that they are engineered to be less soluble and to have a longer duration in the blood, acting between meals to regulate blood glucose (released by the liver). Several formulations are now available with varying pharmacokinetics: protamine zinc insulin, NPH insulin, semilente, lente, and ultralente (57). A new long acting insulin analogue called insulin glargine has been developed that has improved pharmacokinetics over the older formulations (65-67). NPH insulin, for example, results in a peak effect within 3-6 hours, before returning to baseline in 16 hours. By contrast, insulin glargine plateaus at a concentration 2 to 3 times lower than NPH within 6-8 hours, remains essentially unchanged for 25 hours, and is unaffected by injection site (65;67). The advantages of insulin glargine include less

variability in blood glucose, lower noctural frequency of low blood sugars, fall in glycosylated haemoglobin, and a reduction in the rise in blood glucose seen in early morning (65;68-71). The only disadvantage of glargine is some injection site pain, presumably due to the acidic pH of this formulation (69).

In summary, recombinant DNA technology has made it possible to design insulin formulations with improved pharmacokinetics that more closely mimic the normal physiology of people without diabetes (57). However, these formulations need to be administered multiple times a day in order to be effective and blood glucose values need to be carefully monitored (13). Advances in insulin administration and blood glucose monitoring may make it easier for people with diabetes to achieve their management goals (46;72).

ii) insulin administration and blood glucose monitoring

The Diabetes Control and Complications Trial (DCCT) demonstrated that improvement in glycemic control, through intensive insulin therapy and self-monitoring of blood glucose, significantly reduced microvascular complications of diabetes (13). Intensive insulin therapy refers to multiple daily injections (4 or more) or insulin pump use along with blood glucose monitoring 4 or more times a day. Several devises have been designed to make this goal more attainable for the diabetic patient. Insulin can now be administered subcutaneously through needles, pens, pumps, jet injectors, and implantable devises (72). The choice of which devise to use is really up to the individual as each has its pros and cons. The pump for example is very costly (approximately $4500), with supplies ranging from $100 to $150/month. On the other hand, the pump offers patients with type 1 diabetes extreme lifestyle flexibility, for example they do not

have to worry about an insulin peak in the night as the pump continuously delivers a set amount of insulin to keep blood sugar in check during the night (45).

Alternative routes of insulin delivery that are being explored are through the lungs (aerosolized insulin) and through the skin (ultrasound-mediated transdermal delivery) (72). Both offer the advantage of eliminating the pain associated with multiple injections which could potentially increase patient compliance to intensive management regimes and improve treatment outcome. Aerosolized insulin has been shown to be as effective as subcutaneous insulin delivery in controlling blood glucose levels in humans (73). Inhaled insulin is currently in clinical trials in the states and may be available soon for the treatment of diabetes. Low-frequency ultrasound has been shown to allow insulin to diffuse through the skin (74). This has been shown to be safe and effective in human cadavers and rat studies and pending the results of clinical trials may be another option for people with diabetes in the future (72).

Blood glucose monitoring is also an important part of intensive diabetes management (46). It allows the assessment and fine tuning of the insulin regime by the patient in conjunction with the diabetes care team. Advancements in blood glucose monitoring have also taken place to make the life of the person affected by diabetes easier. Options that are now available include the traditional fingerstick testing, alternative-site testing, and continuous blood glucose monitoring (46). Alternative-site testing on the forearms and legs allows people with diabetes to give their fingers a break. These testers often require less blood and are virtually painless. Continuous blood glucose monitoring systems are currently available on the market (the MiniMed Continuous Glucose Monitoring System, and the GlucoWatch). Both of these systems

monitor interstitial fluid, which directly reflects glucose concentrations in the blood (75). The MiniMed devise provides much more information with up to 288 glucose measurements per day for up to 3 days (76). However, the current version does not display real-time glucose values. Instead, the monitor and sensor are worn for 3 days, after which the sensor is removed and stored data downloaded to a computer. Furthermore, at the same time, blood glucose needs to be checked with a standard meter to correlate readings with that of the continuous monitoring devise. This type of monitoring devise can be very useful in getting the patient on a good treatment regime in the clinic, when first diagnosed or when problems arise, but it is not very practical for daily use in its current form (77). The GlucoWatch, however, does provide real-time blood glucose data but it is expensive and the sensor pads have to be changed frequently.

These advancements in insulin formulations, administration, and blood glucose monitoring have certainly made it easier for patients to stick to intensive management protocols, with better outcomes in terms of improved glycemic control with fewer lows and improvements in glycosylated hemoglobin values (45;46;57). However, intensive management is still an arduous task despite these advances and not everyone can afford the latest technology. The only way near normal physiology can be approached is with a closed loop system that both detects blood glucose levels on a continuous basis and delivers insulin appropriately. The pancreas or more specifically the β-cell is well adept at performing this function and although mechanical devises may come close they currently do not provide the level of glycemic control that a β-cell can.

iii) engineering non-beta cells to secrete insulin

One strategy for the treatment of type 1 diabetes involves engineering non-beta cells to secrete insulin through gene therapy. Gene therapy holds several advantages over cell replacement therapy (islet or β-cell line transplantation) in that it does not rely on a limited supply of human organs as a source of tissue for transplantation, nor would it require chronic immunosuppression. However, getting a non-beta cell to secrete insulin in response to glucose has proven to be a difficult task. β-cells have many unique features that make them adept at performing their task of sensing glucose levels in the blood and releasing insulin accordingly (Figure 1-1) (78). β-cells express glucose transporter 2 (GLUT2), which permits rapid glucose uptake (79). Once inside the cell glucose becomes phosphorylated by glucokinase to form glucose 6-phosphate, another important feature of the glucose sensory machinery of the β-cell (80). Unlike most other mammalian cell-types, pancreatic β-cells express low lactate dehydrogenase levels and high pyruvate carboxylase activity (81;82). These two properties can explain why virtually all pyruvate that is formed from glucose 6-phosphate enters the Krebs cycle and why β-cells produce little lactate. Tight coupling between glycolysis and mitochondrial oxidation has been considered to be crucial for more distal steps in the signal generation in β-cells (83;84). An acute rise in extracellular glucose concentrations induces a dose dependent increase in the adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratio (85). This shift in the adenine nucleotide pool then stimulates the closure of ATP-sensitive potassium channels that results in membrane depolarization and opening of calcium channels (86;87). Calcium influx at this point results in the release of insulin from storage granules (87).

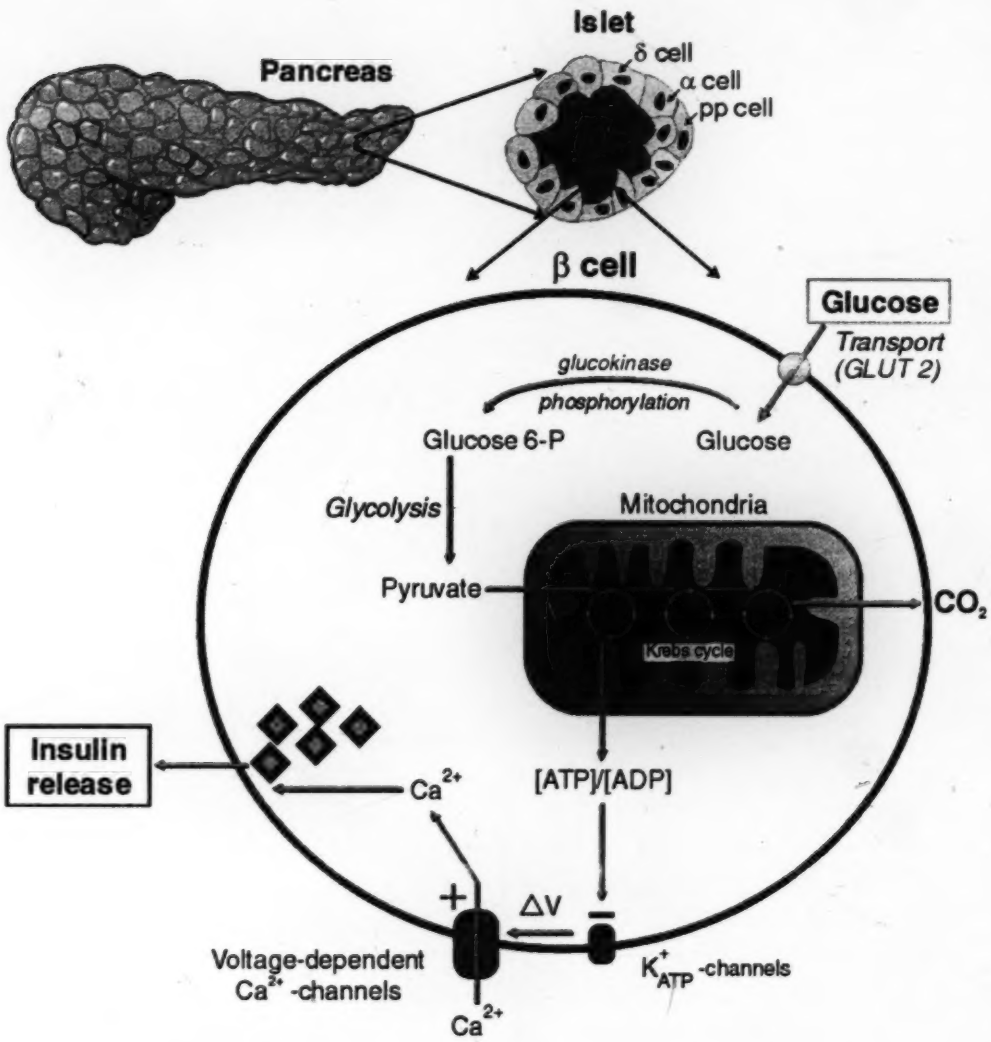

Figure 1-1. The beta (β)-cell is one of the major cell types found in the islets of Langerhans of the pancreas that is involved in regulating blood glucose control through the secretion of insulin in response to fluctuations in blood glucose. The β-cell has many features that make it adept at performing its specialized function which makes it challenging to engineer non-beta cell types to perform the same function and to be used as a replacement for β-cells that are destroyed in type 1 diabetes. Furthermore, islets are multi-cellular structures with many different cell types, beside β-cells, that are also involved in the strict regulation of blood glucose (alpha (α)-cell, glucagon; delta (δ)-cell, somatostatin; polypeptide (pp)-cell, polypeptide) (78).

Many different tissue types have been studied for their ability to express the insulin gene including: liver, muscle, pituitary, hematopoietic stem cells, fibroblasts, and exocrine glands of the gastrointestinal tract (88-92). The main challenge has been to achieve glucose-dependent insulin secretion in non-β-cell types. Both intestinal K-cells and hepatocytes have shown the most promise in meeting this challenge (54;55).

The K-cell, found in the stomach, duodenum, and jejunum, is responsible for the glucose sensitive release of a hormone known as glucose-dependent insulinotropic polypeptide (GIP) which in turn causes the release of insulin (93-95). These K-cells express glucokinase, a very important feature for glucose sensing that is also found in β-cells (54;80). Tumor-derived K-cell lines have been created with the ability to produce human insulin by providing the cells with the human insulin gene linked to the 5'-regulatory region of the gene encoding GIP (54). Transgenic mice have also been created that express this transgene and produce human insulin in their gut K-cells (54). This insulin protected the mice from developing diabetes and maintained glucose tolerance after destruction of the native insulin-producing β-cells. Hepatocytes have also been engineered to produce human insulin and restore euglycemia in diabetic rats (55). The challenge for this technology in the future will be the delivery of the transgene construct to target tissue in larger animal models and humans and to show that it can restore metabolic control in these models in a stable and safe manner.

iv) pancreas and islet transplantation

Pancreas and islet cell transplantation have been used in the clinic for decades with improved success rates for both over time (96;97). Pancreas transplantation has been shown to restore full metabolic control, the ultimate goal of any treatment plan

for diabetes, however, this procedure has many limitations (96;98). Whole and partial pancreas transplants are surgically complex and are associated with significant mortality and morbidity (99). For these reasons pancreas transplantation is limited to patients also in need of a kidney for the treatment of end-stage diabetic nephropathy (100).

The goal of cell replacement therapy is to be able to offer early intervention for the restoration of blood glucose control and the reversal or prevention of chronic complications (101). In type 1 diabetes, only the pancreatic β-cells, found in the islets of Langerhans, are targeted for destruction by the autoimmune process (11). The islets only make up approximately 1% of the whole pancreas, therefore, one approach is to isolate the islets from the pancreas (the portion that is required to restore glycemic control) and just transplant them (Figure 1-1) (101). Islet transplantation offers many advantages over whole pancreas transplantation including: no surgery is required for the implantation of islets, there is a high degree of safety, with no morbidity, islets can be stored in culture or cryopreserved for banking, and islets can be immunomodulated and/or encapsulated to protect them from immune rejection (53). The biggest challenges for islet transplantation have been: 1) to reproducibly obtain high yields of good quality islets from brain dead organ donors, 2) transplantation of an adequate amount of islets that fully restore glycemic control, 3) delayed vascularization, rejection, and/or recurrence of autoimmune disease, and 4) use of steroid immunosuppression with toxic affects on islets (53). Due to these challenges islet transplantation success prior to the year 2000 was reported to be very low with only 8% of individuals treated being free from the need of insulin therapy at one year (102).

A tremendous boost in the success rate was reported using what has come to be known as the Edmonton protocol with 100% of patients insulin free after one year (50). This protocol involves the transplantation of type 1 diabetic patients, with a history of severe hypoglycemia and metabolic instability, with freshly isolated islets (mass of 11,547±1604 islet equivalents per kilogram body weight) in conjunction with a glucocorticoid-free immunosuppressive regimen consisting of sirolimus, tacrolimus, and daclizumab (monoclonal antibody against the interleukin-2 receptor) (50). Islets were prepared in xenoprotein-free medium, and transplanted immediately into the renal portal vein of the liver. The key features of this protocol implicated in its success include the delivery of an adequate amount of freshly isolated islet tissue (requiring 2 and sometimes 3 donor pancreases) in conjunction with a steroid-free immunosuppression, highly effective at preventing allograft rejection and autoimmunity, with no toxic side effects on islets. The Edmonton protocol has now been successfully reproduced by other centers with several key improvements being made (53). One of the biggest drawbacks of the Edmonton protocol is the requirement for at least 2 donor pancreases, and sometimes more, in order to procure an adequate supply of insulin producing islet tissue to reverse the diabetic state. This is a major limitation to this procedure given the shortage of human pancreases available for transplantation.

One major advancement since the initial report of the Edmonton protocol is the success the University of Minnesota group has achieved with single-donor islet transplantation (103). Their success is attributed to their ability to isolate islets with a high degree of potency, possibly due to the use of non-ficoll-based iodixanol density grandients for islet purification together with the use of perfluorodecalin for pancreas

transportation prior to isolation. The University of Pennsylvania was also successful at isolating and using islets from non-heart beating donors, thus expanding the donor pool (104). The use of rescue gradients, developed by the University of Miami, has also proven successful at recovering trapped islets to improve islet yield during the isolation. All of these new developments in islet transplantation in recent years, beginning with the Edmonton protocol, have helped to propel the field forward with a promising future for islet transplantation for the treatment of type 1 diabetes. However, despite recent advances waiting lists for this procedure are very long and even very brittle diabetics whose life depends on this procedure have to wait due to the shortage of human pancreases from which islets can be procured for transplantation. Secondly, the ultimate goal of islet transplantation is to be able to offer the procedure to young, even newly diagnosed, type 1 diabetic patients in order to restore their blood glucose control early enough to prevent the devastating complications of diabetes. However, currently the risks associated with a life-time of immunosuppression outweigh the benefits of the procedure. For these reasons the goal of current research is focused on finding an unlimited supply of tissue for islet transplantation that can be transplanted in such a way that systemic immunosuppression is not required.

I-B) MEETING THE CHALLENGES OF ISLET TRANSPLANTATION

1. Potential Sources of Tissue for Islet Transplantation

a) Living islet donors

Current islet transplantation protocols rely on cadaveric pancreas donors as a source of islets for transplantation (53). At present the pool of cadaveric pancreata

available for islet isolation and transplantation is limited and alternative sources must be found in order to allow for the widespread use of islet transplantation for the treatment of type 1 diabetes. Additionally, some countries, such as Japan, do not allow procurement of organs from their dead. Some centers have had success using living donors for simultaneous pancreas and kidney transplantation (105-107). This procedure involves the removal of a portion of the living donor pancreas for transplantation into the diabetic recipient. This technique could conceivably be extended to islet transplantation. Procurement of organs from living donors is advantageous in several ways. First of all the quality of the organ isolated from a living donor should be greatly improved as compared to an organ isolated from a brain-dead donor (108). Secondly, HLA matching between donor and recipient can occur if the donor is a living relative, for example. Obtaining a closer immunologic match may reduce the amount of immunosuppression required and may improve the function and lifetime of the transplanted organ (109). Finally, such an approach to organ procurement offers the advantage of reducing waiting time and possibly percentage of deaths of people on the transplant list. Removal of a portion of the pancreas of a healthy individual is not without consequence, however. It has been shown that although people can live with only a portion of their pancreas they are at increased risk of developing type 2 diabetes. Other risks include those associated with any major operation. Due to the risks associated with such a procedure it does not appear that living pancreas donors will be a main source of tissue for islet transplantation.

b) Beta cell lines

One potential source of tissue could be found through the development of pancreatic β-cell lines (110). The formation of a β-cell line requires the creation of an

immortalized β-cell that has been oncogenically transformed. For example, βTC cell lines have been created using transgenic technology whereby transgenic mice harboring the SV40 T-antigen driven by the insulin gene enhancer-promoter region develop heritable β-cell tumors (111-114). The mouse β-cell lines have been reported to produce insulin in amounts comparable to normal islets and release insulin in response to physiological stimuli (110). These cell lines also normalize glycemia in diabetic mice (115). However, this technology is not without its challenges. β-cell lines have been shown to dedifferentiate during proliferation in culture leading to such deviations as insulin secretion to subphysiological glucose levels (116;117). A second problem is the regulation of the proliferation of these cells *in vivo* (110). Unregulated β-cell growth can lead to severe hypoglycemia and death of the recipient. More work needs to be done in this area to create a human β-cell line that functions in a manner similar to normal human β-cells in its ability to respond to fluctuations in glucose and at the same time is highly regulated in its growth such that it does not cause tumors in the recipient.

c) Stem cells

One potential source of insulin secreting tissue for transplantation into patients with type 1 diabetes may come from stem cells (118). Stem cells are self-renewing elements that can generate the many cell types in the body. They are found in adult and fetal tissues, but the stem cells with the widest developmental potential are derived from an early stage of the mammalian embryo and are called embryonic stem (ES) cells. ES cells have been shown to differentiate *in vitro* into many different cell types including pancreatic islet-like structures (119-125). Lumelsky *et al.*, operating under the assumption that a strategy used to generate neurons would lead to the development of

islet-like structures, cultured mouse ES cells *in vitro* under conditions that enriched in cells expressing the neural stem cell marker nestin (125). These nestin positive cells were further differentiated into structures morphologically resembling islets. However, much controversy surrounds these observations as the resulting islet-like structures had very low cellular insulin content and failed to normalize diabetic mice. Furthermore, reports that insulin immunoreactivity in ES cell cultures is due to insulin uptake from the media, rather than actual insulin production by the ES cells themselves, calls to question whether or not the above mentioned protocol can actually generate islet-like structures (126). However, more studies have improved upon the original protocol and have been able to generate cells that can correct hyperglycemia in diabetic animals (127;128). It has been demonstrated that insulin-producing clusters can also be obtained from human ES cells (129). The authors of that paper report the development, from ES cells, of cell clusters that expressed insulin, glucagon, and somatostatin. However, no mention was made of the amount of insulin content in these cells or whether or not they possessed the ability to normalize blood glucose in diabetic nude mice. Due in part to the controversy surrounding the isolation of ES cells from human embryos, research in this area has also focused on the development of insulin secreting cells from adult stem cells of the pancreas and other organs. Several groups have reported the successful isolation and differentiation of stem cells derived from adult pancreatic ductal structures expressing endocrine hormones (130-135). Currently, it is thought that duct, acinar, and islet cells all contain cell populations capable of differentiation, trans-differentiation (differentiation along a pathway not normally followed), or de-differentiation into cells that have the potential to become endocrine cells (136). These adult stem cells are cultured for the

enrichment of multi-cell, islet-like structures which are then further matured *in vivo* (136). These islet-like structures were found to be capable of reversing the diabetic state in NOD mice within a week (132). However, *in vivo* maturation of these islet-like structures derived from adult stem cells appeared to be dependent on the site of transplantation with kidney or spleen being superior sites for maturation as compared to muscle (132). An interesting observation in these NOD mice was that there was no incidence of autoimmune recurrence. This suggests that stem cell derived islet-like cells may be less immunogenic, requiring less or no immunosuppression. Although stem cell technology has a long way to go before making it to the clinic for the treatment of patients with type 1 diabetes, initial studies in this area seem very promising and exciting.

d) Xenotransplantation

Xenotransplantation, or transplantation of tissue from one species to another, for example from animal to human, offers a potential solution to the tissue supply problem encountered in islet transplantation. Porcine and bovine islets as well as fish-brockman bodies are all being considered potential sources of tissue for human islet transplantation (137-139). Using pigs, for example, as a source of tissue for islet transplantation offers the advantages of being inexpensive, readily available, ethically acceptable, can be housed in pathogen-free environments and their islets exhibit morphological and physiological characteristics that are similar to our own islets (140). Porcine insulin is also structurally similar to human insulin and has been used for the treatment of type 1 diabetes for decades. Additionally, it has been shown that neonatal porcine islets are the best candidate for eventual transplantation into humans (137). Adult porcine islets are fragile and difficult to maintain in tissue culture and fetal porcine

islets exhibit poor insulin secretory response to glucose (141-143). Neonatal porcine islets, however, can be isolated in large numbers, show a potential for growth *in vitro* and *in vivo*, show excellent ability to respond to glucose challenge and are capable of restoring euglycemia in diabetic mice (144). One major drawback for using pigs as a source of tissue for islet transplantation is the risk of transmission of porcine endogenous retrovirus (PERV), a virus that is in the germline of every pig (145). Although studies have shown that PERV can infect human cells *in vitro* there is no evidence of transmission in human recipients of pig tissue *in vivo* (145-147). Due to the potential introduction of this virus in the human population studies of pig-to-human transplantation are proceeding with caution. Another major obstacle to overcome in order for xenotransplantation to be able to move forward is the issue of hyperacute rejection of xenografts in humans. This process is believed to be initiated by naturally occurring xenoreactive antibodies in human sera that bind to antigens present on the cells within the xenograft. Antibody binding in turn activates complement, which rapidly destroys the transplanted tissue through an intense inflammatory response (148). The most important target for these naturally occurring xenoreactive antibodies was found to be the terminal carbohydrate galactose α(1,3)-galactose (Galα(1,3)Gal) (149-151). One strategy for preventing hyperacute rejection of porcine islets is to immunoisolate them by encapsulation (discussed in more detail in the next section) (137). Briefly, the idea of such a devise is to allow the diffusion of low molecular weight molecules (glucose, insulin) yet exclude cellular contact between donor and host cells as well as the passage of large molecular weight molecules (immunoglobulins). Microencapsulated neonatal porcine islets also show improved islet recovery and function following extended periods

of tissue culture and cryopreservation (137). Tissue culture allows the maturation of neonatal porcine islets *in vitro* and cryopreservation makes it possible to pool several preparations of porcine islets for transplantation of a large number of functional islets. Another obstacle to overcome is that of autoimmune recurrence which is a major problem in allograft transplantation (MHC mismatched members of the same species). This may potentially not be a concern for xenotransplantation as it has been shown that bovine islets, for example, are less susceptible than human islets to the effects of human cytokines, a major player in autoimmunity (152). However, other autoimmune mechanisms could still potentially destroy the xenograft (CTL killing).

Finally, the use of fish Brockmann bodies holds an advantage over neonatal porcine islets in that they do not require a lengthy isolation procedure and can be easily microdissected (153). Fish Brockmann bodies, like neonatal porcine islets, are subject to hyperacute rejection. Microencapsulation of fish Brockmann bodies has been shown to be possible and encapsulated Brockmann bodies are capable of restoring euglycemia in diabetic mice (154). Additionally, fish Brockmann bodies appear to hold an advantage over pig islets in that no endogenous retrovirus has been identified in fish that can potentially be transmitted to the human population. Studies are currently underway to engineer fish Brockmann bodies that secrete human insulin to make them more clinically applicable (155).

2. Strategies for Overcoming the Requirement for Immunosuppression

a) Encapsulation

One attractive means of protecting transplanted islets from immune attack is through immune isolation. The main goal of this technology is to develop a device that

allows the passage of small molecules such as glucose, insulin, nutrients, oxygen, and waste products to maintain highly functional and viable islets while at the same time excluding passage of larger immune cells and molecules that might destroy the islets (Figure 1-2). Immune isolation devises should also be highly biocompatible such that cells do not stick to the outside of the devise and limit the diffusion of small molecules needed for the functioning of the islet cells. Several devises have been developed and tested in animal models including: vascular perfusion devises, macrocapsules, and microcapsules (Figure 1-2) (156).

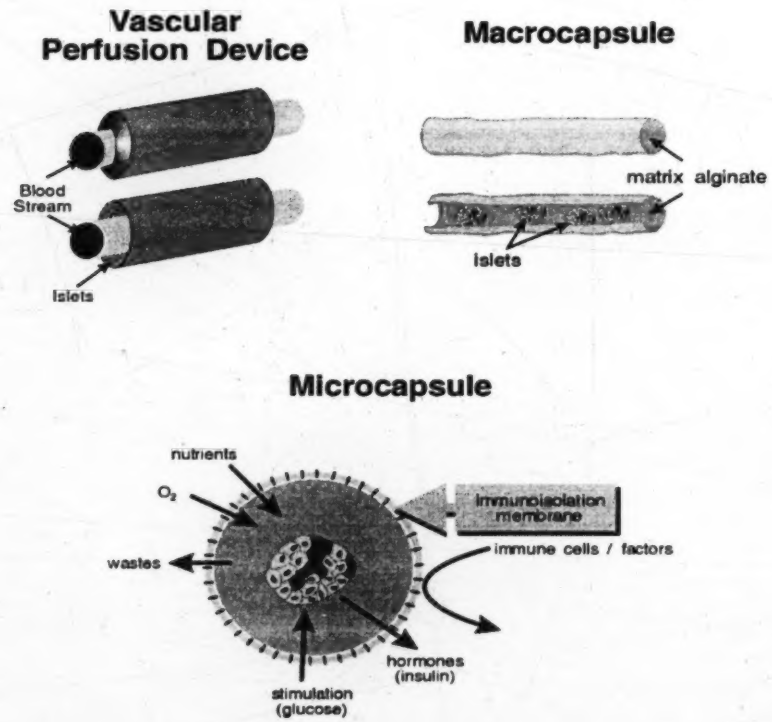

Figure 1-2. Immune isolation devises are designed to protect islets by excluding immune cells and large immune complexes but at the same time permitting the passage of nutrients, oxygen (O_2), waste products, insulin, and glucose so encapsulated islets can function normally. Shown here are three examples of immune isolation devises: the vascular perfusion device, macrocapsule, and microcapsule.

Vascular perfusion devises have a hollow internal capillary tube that is connected to the vascular system to allow blood to flow through its lumen (156). Outside the internal tube is attached a chamber where the tissue to be transplanted is placed (Figure 1-2). This type of devise allows close contact between the graft and the blood stream which allows the implanted tissue to be well nourished. Pig islets housed in this devise have been able to achieve and maintain long-term normoglycemia in canine recipients (157). However, despite initial successes with this devise several challenges limit its current clinical applicability. First of all, because of the small size of the devise only a small amount of tissue can be implanted into a single devise. Devises that have been lengthened failed to operate properly due to blood clotting. A second issue is that these devises do not allow for optimal glycemic control. Finally, implantation of these devises is not simple and requires major vascular surgery (158).

Macroencapsulation involves the implantation of tissue into a single extravascular diffusion chamber that may be tubular or planar (Figure 1-2) (156). Porcine, bovine, and canine islets implanted into these chambers have all been able to restore blood glucose control in diabetic rats (158). Problems with these diffusion chambers include: membrane breakage, overgrowth (poor biocompatibility), and diffusional issues such as necrosis of implanted tissue and accumulation of waste due to the size of the devise (158).

Microencapsulation involves enclosing individual islets in individual spherical shaped capsules (Figure 2-1) (156). This approach to immune isolation offers several advantages over macroencapsulation. First of all, the spherical shape of the microcapsule provides a better surface to volume ratio than the disk or tube shapes of the

macrocapsules. The advantage of having an improved surface to volume ratio is that there is better diffusion from the capsule, reducing the amount of cell necrosis within the capsule and waste accumulation. A second major advantage to microencapsulation over the other immune isolation devises is that each islet is contained in its own capsule; therefore, if one capsule breaks the entire transplant does not fail (156). Microencapsulation has faced problems in the past with low biocompatibility, fibroblast overgrowth, as well as low stability (159;160). These problems have been addressed with improvements in capsule design (alginate-polylysine, and barium-alginate). Alginate-polylysine microcapsules have been shown to prolong islet xenograft survival in both small (mouse) and large (dog and monkey) animal models (161-163). The alginate-polylysine microcapsule has also been used in human clinical trials where insulin independence was achieved up to 9 months after transplantation (164). Despite success with this technology the capsule barrier may not be enough to prevent autoimmune recurrence which is primarily mediated by cytokines that could get through the capsule. However, microencapsulation of genetically modified islets may have a powerful synergistic effect in preventing both graft rejection and autoimmune destruction.

b) Co-stimulation blockade

Islet transplantation from a human cadaveric source to a type 1 diabetic recipient involves transplantation across a major histocomplatibility barrier. Alloreactive T cells in the recipient recognize donor cells as being foreign and become activated to destroy them. T cell activation requires several important signals (Figure 1-3A) (165).

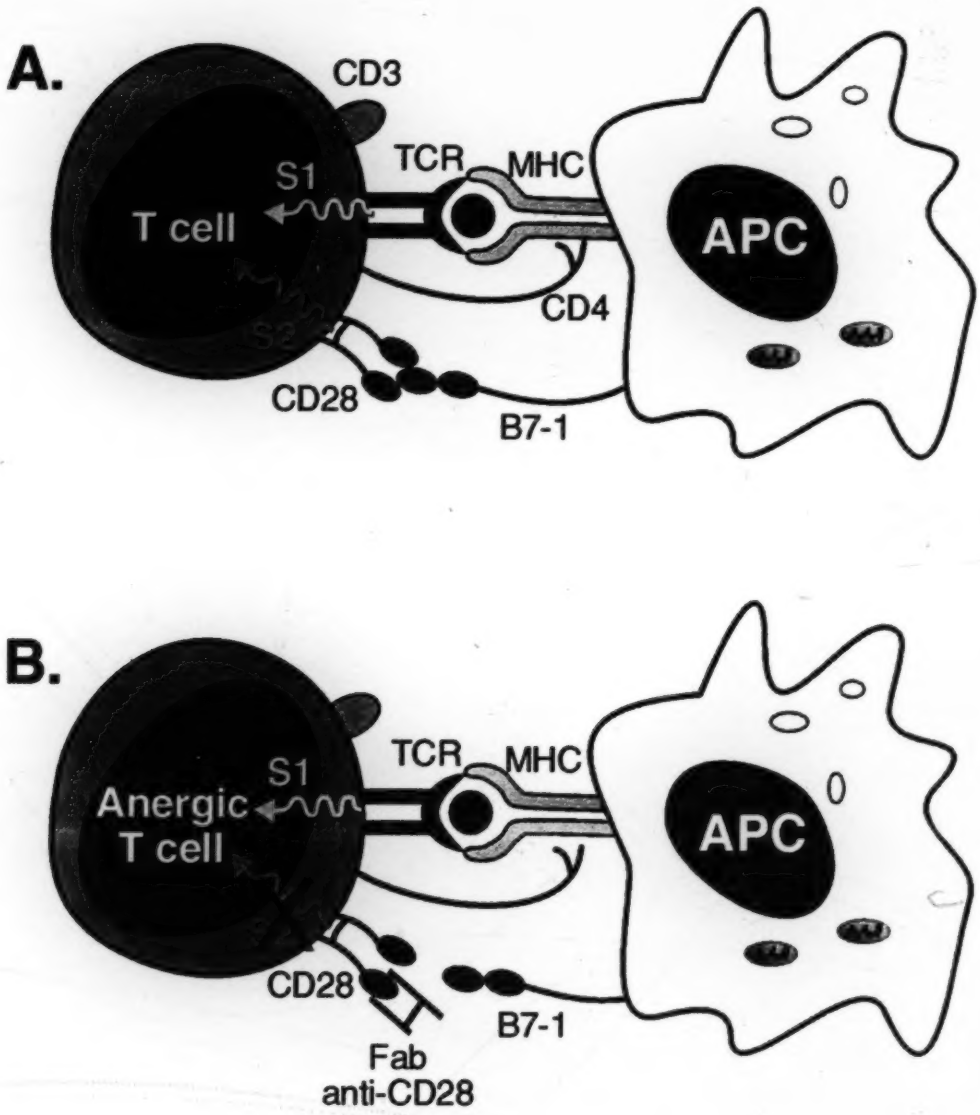

Figure 1-3. T cell activation and co-stimulatory blockade. (A) Naïve T cells become activated when they encounter antigen presented on an antigen presenting cell (APC) through a series of signals: signal 1 (S1), T cell receptor (TCR) interaction with antigen in the context of correct major histocompatibility complex (MHC); and signal 2 (S2), interaction between co-stimulatory molecules (cluster of differentiation [CD]28 and B7-1). (B) Blockade of co-stimulator signal 2 with an antibody directed at a co-stimulatory molecule (CD28) results in an anergic T cell that is unable to respond to antigen.

The first signal for activation is initiated when the T cell receptor (TCR) recognizes antigen in the context of MHC on an APC (dendritic cell, macrophage, or B lymphocyte). The second signal is a co-stimulatory signal that comes from interaction of B7 molecules on the surface of the APC with either CD28 or cytotoxic T lymphocyte antigen-4 (CTLA-4) on the surface of T cells (Figure 1-3A). Both signal one and signal two are required for full T-cell activation. If signal one is initiated in the absence of co-stimulatory signal two then the T-cell becomes anergic and can not respond to foreign antigen (Figure 1-3B) (165). One method of blocking co-simulation is through monoclonal antibody therapy directed at the co-stimulatory molecule. One such antibody, CTLA4-Ig, blocks the interaction of CD28 with the B7 molecule (166;167). Many studies have shown the effectiveness of CTLA4-Ig at prolonging allograft and xenograft survival; however, this therapy was found to be more effective when combined with other tolerance inducing strategies such as bone marrow transplantation, or low-dose immunosuppression (168-171). CTLA4-Ig has also been transfected into islets and myoblasts co-transplanted with islets for the prolongation of graft survival (172;173). A second monoclonal antibody capable of blocking co-stimulation is anti-CD40 ligand that blocks CD40 interaction with its T cell ligand, CD40 ligand, another important co-stimulatory signal for T cell activation (174;175). It was demonstrated that treatment with either CTLA4-Ig or anti-CD40 ligand alone was not enough to prevent the rejection of skin allografts in mice but when used in combination these antibodies had a powerful synergistic effect and were capable of significantly prolonging graft survival (176). At this point it appears that co-stimulatory blockade using one type of antibody alone may not be enough to prevent allograft rejection and autoimmune destruction of the islet graft but perhaps the use of

multiple antibodies against co-stimulatory molecules or the combination of this therapy with others (low does immune suppression and/or bone marrow transplantation) may significantly prolong graft survival. More research on the safety and efficacy of this approach needs to be carried out before advancing to clinical trials.

c) Mixed Chimerism

Myeloablation of recipients through whole body irradiation or chemotherapy followed by donor-specific bone marrow reconstitution establishes mixed chimerism in recipients and is a proven way to induce tolerance to donor grafts (53). Mixed chimerism works through central tolerance mechanisms in that any donor specific T cells that are generated get deleted during negative selection in the thymus (31). Even though highly effective this therapy possess significant risk for the recipient due to graft versus host disease, a reaction that develops when a graft contains immunocompetent T cells that recognize and attack the recipient's cells, and harsh pre-conditioning treatments (irradiation and chemotherapy) (53). Development of less harmful non-myeloablative strategies may make this therapy more clinically applicable for the pre-conditioning of type 1 diabetic patients.

d) Dendritic cells

One of the major cell types involved in antigen presentation and T cell activation is the dendritic cell (DC). Donor DCs are thought to be one of the cell types that are passenger leukocytes in organ transplantation that can survive within the recipient inducing a state of microchimerism. Originally it was thought that DCs play an important role in graft rejection and that islet cells, for example, would be less immunogenic than whole organ transplants as islets would have less passenger

leukocytes (177). However, this is not the case in that islet cell grafts have been shown to be rejected just as rapidly as whole pancreas grafts. Now it is recognized that DCs not only function as potent APCs, but also as potential inducers of T cell tolerance (178;179). It has been shown that immature DCs induce donor-specific T cell hyporeactivity and allograft acceptance, whereas mature DCs activate T cells and cause accelerated graft rejection (180-183). One explanation for this phenomenon is the observed lack of co-stimulatory molecules on immature DCs and elevated co-stimulatory molecules on mature DCs (180;184). In the future it may be possible to isolate and generate immature donor DCs and co-transplant them along with same donor islet grafts to prevent allograft rejection. Autoimmune recurrence may still be an issue with DC therapy. Nevertheless research in this area will help us better understand mechanisms of graft rejection versus tolerance and may help provide a safe and effective way of achieving graft tolerance.

e) Intrathymic administration of donor antigens

The entire T cell population is selected for in the thymus (31). Thymocytes from the bone marrow migrate to the thymus for selection and further maturation into mature CD4 and CD8 positive T-cells. In the thymic cortex thymocytes that bind self MHC are positively selected for while those thymocytes that do not interact with self MHC die by apoptosis. Following positive selection thymocytes then migrate to the thymic medulla where they encounter self-antigen. Any thymocytes with a high affinity receptor for self-antigen are negatively selected and die by apoptosis (31). This mechanism of central tolerance has evolved to protect from harmful self reactive T cells. However, when central or peripheral tolerance breaks down, autoimmunity occurs. It has been demonstrated that intrathymic transplantation of donor antigens is capable of

inducing long-term survival of subsequent islet transplants in animal models (185). Although promising, one major limitation to this therapy in pre-conditioning patients with type 1 diabetes is that they often have thymic atrophy (186). Clinical attempts have yet to reproduce animal findings (187).

f) Transplantation into immune-privileged sites

Immune-privileged sites are regions of the body where grafts of foreign tissue survive for extended periods or even indefinitely, compared to conventional or non-privileged sites (188). Islet allografts have been shown to have extended survival in the testis, brain, and thymus (185;189;190). There are many features of immune-privileged sites that enable the acceptance of grafts: blood-tissue barriers, absence of efferent lymphatics, FasL expression, and secretion of immunosuppressive factors (transforming growth factor-beta [TGF-β]) (188). Constitutive FasL expression, for example, in immune-privileged sites has been shown to engage the Fas receptor on the surface of infiltrating activated T cells causing them to die via apoptosis (191). Originally it was thought that the maintenance of immune-privilege in certain tissues was due to isolation behind immune barriers such as blood tissue barriers and that the immune system was simply ignorant of any antigens in the privileged tissue (192). However, we now know that the immune system does recognize antigens in immune-privileged sites but the immune system is tolerant to these antigens. It is known, for example, that after injection of antigen into the eye, dendritic cells in that region pick up the antigen and migrate to splenic white pulp where antigen-specific regulatory and effector T cells are activated (193-195). The immune system therefore plays an active role in maintaining immune-privilege. Transplantation of islets into immune-privileged sites, however, has

limited clinical applicability. For example, transplantation of islets into the testis would only be possible for male type 1 diabetics (189). Additionally, immune-privileged sites may not be ideal locations for islet function and the restoration of blood glucose control. It has been shown that the Sertoli cell is responsible for maintaining the immune privilege environment of the testis and islet allografts co-transplanted with Sertoli cells beneath the kidney capsule of diabetic mice survive for extended periods as compared to islet allografts transplanted alone (196-199). It may, therefore, be possible to co-transplant islets with Sertoli cells, an immune privileged tissue, into a more clinically relevant site to create a localized area of immune suppression for islet graft survival. Before this can be achieved the optimal conditions for establishing such an immune-privileged site outside the testis must first be established and the mechanism of how Sertoli cells protect themselves and other bystander cells from immune rejection needs to be examined. These studies may uncover mechanisms utilized by Sertoli cells for blocking immune function that can be exploited in the clinic to protect islets and other grafts from rejection.

I-C) THE SERTOLI CELL

Sertoli cells are found within the seminiferous tubules of the testis where their main function is to support the growth and development of new sperm (Figure 1-4) (200). Sertoli cells line the inner lumen of the seminiferous tubule and are closely associated with one another via tight-junctions which form the blood-testis barrier. Sertoli cells have also been shown to be responsible for the maintenance of the immune-privileged environment of the testis which protects sperm from harmful immune reaction (196). It is thought that many features of Sertoli cells make them adept at performing this

function, including the establishment of a blood-testis barrier, the secretion of multiple growth and immunosuppressive factors (Table 1-1), as well as the expression of FasL (201;202). Islet allografts placed within the testis, in the extratubular space (Figure 1-4), survive for extended periods of time (189). Because the immune-privileged region extends outside the blood testis barrier to include the interstitial space, it appears that some sort of Sertoli cell secreted factor may be responsible for maintaining immune privilege. It has been suggested that production of immunosuppressive factors within the seminiferous tubules depends on the stage of the epithelial cycle that the tubules are in (203). For example, tubule segments in stages II-VIII of the seminiferous cycle inhibited peripheral blood lymphocyte proliferation significantly more than stages IX-I. Immunosuppressive activity within the seminiferous tubules and outside the tubules, in the interstitium, was found to be associated with 10, 25, 65, 200, 400 kDa sized proteins. The 25 kDa protein is thought to be TGF-β, an anti-inflammatory protein. Additionally, rat islet allografts have been shown to survive for extended periods of time in the testis provided the testis is placed intra-abdominally (189). A potential reason for the differential function of islets implanted in the scrotal versus the abdominal testis may be that islets function optimally at the higher temperature of the body. Hamster islet xenografts have also been shown to survive for extended periods in the intra-abdominally placed testis provided a short course of cyclosporine is given initially (204;205). Despite successful prolongation of islet graft survival within the testis of rodents, the potential clinical application of these findings is limited. However, the knowledge that it is the Sertoli cell that is responsible for the maintenance of immune-privilege within the testis and the protection of grafts in this site, favors the option of isolating the Sertoli cell from

the testis and co-transplanting it with islets into non-privileged sites to create an immune-privileged site outside the testis with more clinical relevance.

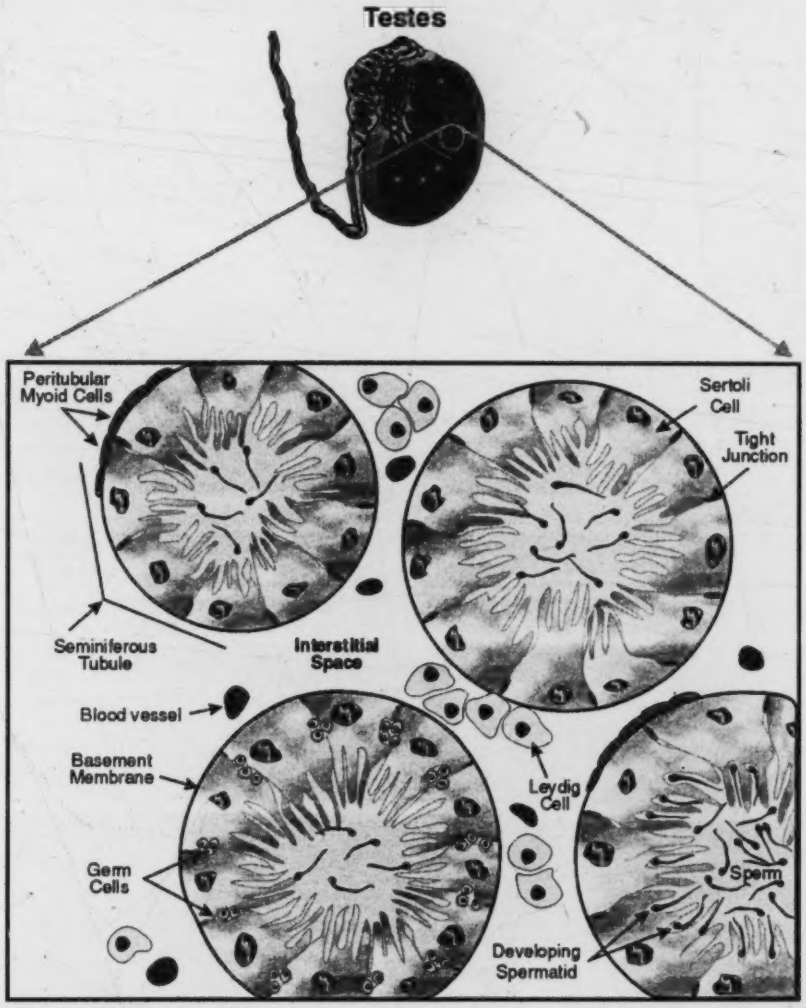

Figure 1-4. Sertoli cells are located within the seminiferous tublules of the testes where they play a major role in spermatogenesis through the secretion of multiple factors that aid in the growth and development of new sperm as well as protect sperm from harmful immune reactions. Sertoli cells are also thought to be responsible for the maintenance of the immune privileged environment of the testes as it has been found that grafts of foreign tissue, transplanted into the interstitial space, survive for extended periods of time (Sertoli Technologies, Inc.).

Table 1-1. Proteins secreted by Sertoli cells. Those in bold may be involved in active immune suppression (201).

Category	Protein	Function
TRANSPORT AND BIOPROTECTION	-transferrin	-iron transport
	-ceruloplasmin	-copper transport
	-saposin	**-binds glycosphingolipids**
	-SGP-2 (clusterin)	-lipid transport, **-complement inhibitor**
	-androgen binding protein	-androgen transport/localization
		-calcium binding protein
	-SPARC	-IGF transport
	-IGF binding proteins	-riboflavin transport
	-riboflavin binding proteins	
PROTEASES AND PROTEASE INHIBITORS	-plasminogen activator	-protease
	-cyclic protein-2	-protease inhibitor
	-cystatin	-protease inhibitor
	-α_2-macroglobulin	-protease inhibitor
	-type IV collagenase	-protease
	-metalloproteinases	-proteases, **cleaves FasL from the membrane, and potentially other receptors**
BASEMENT MEMBRANE	-collagen IV	-extracellular matrix component
	-laminin	-extracellular matrix component
	-proteoglycans	-extracellular matrix component, **bind granzyme B**
HORMONES/ GROWTH FACTORS	-mullerian inhibiting substance	-inhibition of Mullerian duct development
	-inhibin	**-inhibition of FSH release**
	-IGF-1	**-growth factor, immunosuppressive agent**
	-prodynorphin	
	-IL-1	**-mitogen**
	-TGF-β and TGF-α	**-growth factors, immunosuppressive agents**
	-basic fibroblast growth factor	-growth factor
	-Sertoli cell secreted growth factor	-growth factor
	-seminiferous growth factor	-growth factor
	-leydig cell stimulatory activity	
OTHERS	-testins	
	-CMB proteins	
	-sulfated glycoprotein-2	**-complement inhibitor**
	-FasL	**-induces apoptosis**
	-lactate/pyruvate	-energy metabolites

1. Co-transplantation of Islets with Sertoli Cells

The first attempt by Selawry and Cameron to co-transplant islet allografts with Sertoli cells beneath the kidney capsule of diabetic rats showed prolongation of graft survival only when a short course of cyclosporine was given immediately post-transplantation (197). Korbutt *et al.* hypothesized that the reason a short course of cyclosporine was required was due to the fact that the Sertoli cells were not fully functional immediately post-transplantation (198). Korbutt made several key modifications to the Sertoli cell preparation procedure and was able to successfully prolong islet allograft survival beneath the kidney capsule of diabetic rats, without the need for immunosuppression, provided the islets were co-transplanted with an adequate number of testicular cell aggregates. The modifications to the Sertoli cell preparation protocol included the preparation of a less highly purified Sertoli cell preparation along with the introduction of a period of *in vitro* tissue culture in non-tissue culture treated plates to allow the formation of testicular cell aggregates (198). Since these original observations, Sertoli cells have been found to protect islet allografts, xenografts, and even islet isografts transplanted into NOD mice, from autoimmune destruction (199;206-208). The ability of Sertoli cells to protect islets appears to be dependent on Sertoli cell number, with too few cells not providing adequate protection and too many cells resulting in neutrophil chemotaxis and graft destruction (208). It has also been suggested that co-transplanting islets with higher doses of Sertoli cells interferes with islet graft function. Besides co-transplanting islets with Sertoli cells, Kin *et al.* have shown that it is possible to pre-implant Sertoli cells for the creation of an ectopic immune privileged site that could accept and protect islet allografts transplanted at a later time (209). The advantage

of this approach is that it allows the establishment of Sertoli cells under the kidney capsule *in vivo* and gives them time to arrange themselves in tubule-like formations, as they exist in their native testis. This formation is hypothesized to be important for their immuno-protective function. Additionally, co-culture and co-encapsulation of islets with Sertoli cells has been shown to improve islet function and has even been suggested to cause terminally differentiated β-cells to proliferate (210-212). It is thought that Sertoli cells achieve these effects on islets through the secretion of multiple growth factors, for example insulin-like growth factor-I (IGF-1) (201). Therefore, it appears that it may be advantageous to co-culture and then co-encapsulate islets with Sertoli cells prior to transplantation. Co-encapsulation offers the added advantage of keeping the islets in close proximity to Sertoli cells as well as providing an additional immune barrier (213). Despite repeated reports that Sertoli cells are an immune privileged tissue that can protect bystander cells from immune destruction, the mechanism of how Sertoli cells interact with the immune system to achieve this goal remains illusive.

2. Potential Mechanisms of Sertoli Cell-Mediated Protection of Islets

a) Fas ligand

A main way that immune privileged tissue has been shown to limit immune responses is through the constitutive expression of FasL (191). When membrane bound FasL engages the Fas receptor, both highly expressed on activated T lymphocytes, it results in apoptosis or cell death of the Fas-bearing cell (214). This mechanism has evolved to limit the immune response such that when a pathogen is cleared the immune response is dampened and autoimmunity does not occur. A perfect illustration of this is in mice that lack a functional FasL (*gld* mice) or lack the Fas receptor (*lpr* mice). Both

gld and *lpr* mice exhibit lymphoproliferative disorders and eventually get autoimmune disease (215;216). In immune privilege FasL expression has been shown to result in apoptosis of infiltrating T lymphocytes resulting in tissue protection. Immune privilege tissues such as Sertoli cells have also been shown to employ the FasL system to protect itself and other tissue (islets) co-transplanted with it from immune destruction. Bellgrau *et al.* showed that only testis grafts isolated from mice with a functional FasL could protect themselves from rejection (202). Takeda *et al.* extended this observation to show that only FasL expressing testicular grafts could prolong the survival of islet allografts (199). Additionally, co-transplantation of islets with myoblasts genetically engineered to express FasL prolonged islet allograft survival in diabetic mice (217). However, FasL expression by mouse β-cells, achieved either transgenically or by gene transfection, failed to protect islet allografts from rejection (218;219). FasL expression in this case resulted in an inflammatory response and neutrophil chemotaxis. Similar results have been shown when islet isografts are co-transplanted with large amounts of Sertoli cells in NOD mice (208). Sertoli cells in this case exerted both protective and destructive effects on islet grafts in the NOD mouse, depending on the amount of Sertoli cells transplanted and FasL expression. It appears that although Sertoli cell expression of FasL has been shown to induce apoptosis in infiltrating T cells, FasL alone is not enough to protect these cells and other bystander cells from destructive inflammatory responses, possibly mediated by high levels of FasL.

b) Transforming growth factor-beta

TGF-β is an anti-inflammatory cytokine capable of suppressing T-cell, macrophage, natural killer cell, and B cell activity as well as the expression of many

proinflammatory cytokines (220). Treatment of diabetes prone NOD mice with a vector encoding mouse TGF-β1 complementary DNA effectively protected these mice from developing insulitis and diabetes (221). Additionally, subcutaneous injection of TGF-β prevented the proinflammatory response mediated by a colon carcinoma cell line transfected with FasL and protected the cell line from rejection (222). Sertoli cells are known to secrete TGF-β and it is possible that the role of this cytokine is to limit the proinflammatory effect of FasL (223). The importance for TGF-β and not FasL in the immunoprotective role of Sertoli cells was shown in the NOD mouse model where the administration of anti-TGF-β1 antibody to NOD recipients of islet/Sertoli grafts completely abrogated the protective effect of Sertoli cells, resulting in islet graft destruction (224). Similar findings were not observed with the use of an anti-FasL antibody. However, one report showed that transgenic mice whose pancreases were made to express TGF-β failed to be protected from immune destruction when transplanted across a major MHC barrier (225). Still, it seems likely that Sertoli cells use constitutive expression of FasL to induce apoptosis in infiltrating T-cells and the secretion of TGF-β to limit the proinflammatory response, culminating in graft protection.

c) Other Sertoli cell secreted factors

i) secretion of a protein(s) from Sertoli cells that inhibits lymphocyte proliferation

Priming of the immune response involves several phases (165). Allo-specifc naïve T cells must first encounter antigen presented on professional APCs in a major lymphoid organ. T cells receive several signals for activation from the APC: signal 1: interaction between T cell TCR and antigen presented on MHC of the APC,

signal 2: interaction between co-stimulatory molecules on the T cell and APC, signal 3: cytokines (IL-2). Upon receiving these essential signals for activation, the naïve T-cell proliferates and differentiates into effector and memory cells. Therefore, as discussed previously, anything that blocks the various signals for T-cell activation or that can inhibit T-cell proliferation in response to an allogeneic antigen, can effectively stop the differentiation of these cells into effector/killer cells and thus prevent graft rejection. Cultured Sertoli cells have been shown to secrete a factor or multiple factors that can effectively inhibit phytolectin-stimulated lymphocyte proliferation in a dose dependent manner (226-228). This effect could also be extended to cell lines of non-lymphoid origin and from various species indicating that Sertoli cells have a generalized effect on cell proliferation. Factors secreted by Sertoli cells, however, were shown to have both a stimulatory as well as an inhibitory effect on lymphocyte proliferation depending on dose (226). Low doses of Sertoli cell proteins resulted in enhanced proliferative responses and high doses in inhibition. It was suggested that the inhibitory activity may be diluted away sooner leaving only stimulatory activity. The mechanism for the inhibitory effect of Sertoli secreted proteins has been attributed to the inhibition of IL-2 production and responsiveness by a factor whose release was found to be dependent on temperature (37^{o}C) and on the presence of follicle stimulating hormone (FSH) in culture medium (227). FSH has been shown to down regulate the production of TGF-β by Sertoli cells, potentially eliminating the possibility that this unknown secreted factor is in fact TGF-β. The factor responsible for the inhibition of lymphocyte proliferation has yet to be identified. However, studies have shown that IGF-1, known to be secreted by Sertoli cells, has the ability to inhibit IL2-induced proliferation of lymphocytes (229). Whether

or not IGF-1 alone or in combination with other Sertoli secreted factors is responsible for the inhibition of lymphocyte proliferation remains to be seen. These studies need to be extended to characterize the effect of Sertoli secreted proteins on lymphocyte proliferation in response to an allogeneic stimulus and not just mitogen stimulation as has been previously described.

ii) secretion of a protein(s) from Sertoli cells that inhibits cytotoxic T lymphocyte killing

CTLs are one of the major cytotoxic effector cells of the immune system. Two main mechanisms of CTL-mediated target cell lysis have been extensively studied: receptor- and granule-mediated (230-233). The principle receptor pathway that has been described in CTLs involves the Fas-FasL system. FasL on activated CTLs engages the Fas receptor on target cells, causing them to undergo apoptotic cell death (214). However, as described previously, this system is thought to be more important in controlling the CTL immune response, although it can mediate target cell lysis under certain conditions (234). The major mechanism for CTL-mediated destruction of target cells involves the delivery of cytolytic effector molecules (235). These proteins, including perforin and granzymes, are stored in the cytoplasmic granules of CTLs and are released during CTL-mediated target cell lysis (236;237). The main CTL granule component implicated in rapid DNA fragmentation, a hallmark feature of apoptosis, is granzyme B (238-241). Upon encounter with a target cell, CTL releases granzyme B and perforin. Granzyme B binds its receptor, the mannose-6 phosphate/insulin-like growth factor-II (M6P/IGF-II) receptor, and along with perforin is endocytosed by the target cell (242). Once inside the target cell, granzyme B is released form the endocytic vesicle by perforin into the cell's cytoplasm where it targets several substrates (caspases and Bid) to

initiate the apoptotic program and cause target cell death (Figure 1-5) (233;243-245). More specifically, granzyme B has been shown to initiate the apoptotic signal of a cell by cleaving caspases at aspartic acid residues (244;246-252). Caspases are synthesized as inactive precursors and cleavage by granzyme B produces two subunits, which together form the active protease (253). Once activated, caspases are able to propagate the apoptotic signal by cleaving and activating other substrates involved in DNA fragmentation (254).

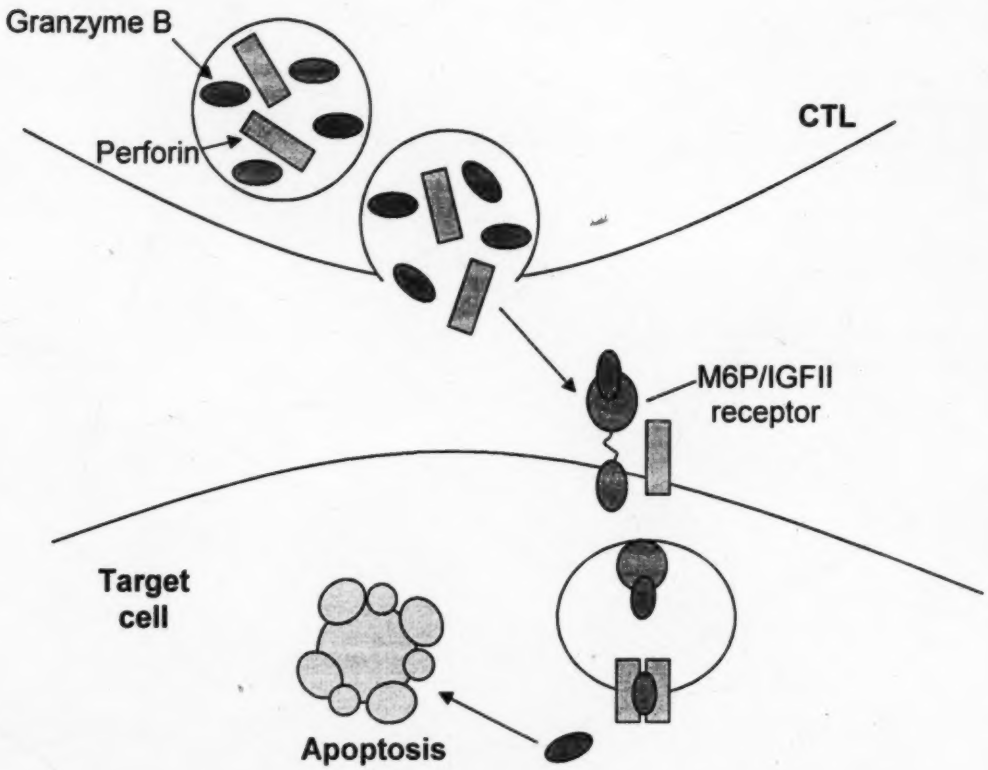

Figure 1-5. Cytotoxic T lymphocyte (CTL)-mediated target cell destruction. When the CTL comes into contact with the target cell, it releases granzyme B and perforin (stored in granules of the CTL). Granzyme B binds its receptor, the mannose-6 phosphate/insulin-like growth factor II (M6P/IGFII) receptor, on the surface of the target cell and becomes endocytosed along with perforin, required for releasing granzyme B from the endocytic vesicle. Once inside the target cell granzyme B targets several substrates culminating in target cell death via apoptosis (233).

Interestingly, Sertoli cells have been shown to secrete multiple ligands for the M6P/IGF-II receptor, including approximately 10 mannose 6-phosphorylated (M6P)-glycoproteins identified by two-dimensional electrophoresis (255). Sertoli cells were also found to express IGF-II messenger RNA. One of the M6P-glycoproteins was identified as procathepsin L, a protein secreted by tumor cells that helps with tumor survival (256;257). Some other M6P-glycoproteins secreted by Sertoli cells include TGF-β and saposin. The latent form of TGF-β has been previously shown to bind to the M6P/IGF-II receptor and become biologically active (258). This cytokine, discussed previously, has many known immunosuppressive functions, but its main role is thought to be as an anti-inflammatory agent (220). Recently, it has been shown that Sertoli-secreted M6P-glycoproteins bind the M6P/IGF-II receptor on the surface of spermatogonia resulting in changes in gene expression in the developing sperm (255). This may be how Sertoli cells communicate with spermatogonia to regulate growth and development of sperm. One avenue that has yet to be explored is whether or not Sertoli cell secreted M6P-glycoproteins inhibit CTL killing of target cells by blocking the binding of granzyme B to its receptor, the M6P/IGF-II receptor. This may potentially be another mechanism employed by Sertoli cells to protect themselves and other bystander cells from harmful immune effector mechanisms.

Human proteinase inhibitor-9 (PI-9) and mouse serine proteinase inhibitor-6 (SPI-6) are both potent inhibitors of granzyme B that inhibit by binding irreversibly (259). Cytplasmic expression of these inhibitors protects cells from granzyme B-mediated apoptosis (259). Both CTLs and natural killer cells have cytoplasmic PI-9 that protects them from any misdirected granzyme B (260). This helps these killer cells to be resistant

to their own cytotoxins so that they do not undergo premature apoptosis before they have performed their function. PI-9 has also recently been found in dendritic cells and in immune-privileged sites such as the placenta, ovary, eye, and the testis, where it potentially helps protect these tissues from immune effector molecules (261;262). Additionally, tumors have also been shown to be able to evade perforin/granzyme B-dependent killing through the expression of PI-9 (263). Even though PI-9 has been found in Sertoli cells what remains to be determined is whether or not it is secreted for the localized inhibition of granzyme B and the protection of bystander cells, such as islets, from CTL killing.

I-D) OJECTIVES AND GENERAL OUTLINE

The main objective of this thesis is to explore methods that will further optimize islet/Sertoli cell co-transplant conditions as well as to elucidate the mechanism of how Sertoli cells interact with the immune system to protect themselves and islets co-transplanted with them from immune destruction.

Our lab has shown previously that co-transplantation of islet allografts with Sertoli cells beneath the kidney capsule of diabetic rats can prolong islet allograft survival (>95 days in 100% of animals) (198). The original goal of Chapter II was to reproduce these findings in a mouse model in order to examine the mechanism of how Sertoli cells might be protecting islet allografts. The choice of mouse over rat was made due to the availability of mouse monoclonal antibodies and knockout mice that could be used in the examination of the mechanism of Sertoli cell protection. For example, TGF-β has been implicated in the mechanism of how Sertoli cells protect islets from

autoimmune destruction in the NOD mouse model using monoclonal antibodies directed at TGF-β1 (224). We were interested in examining if this is also the case in the alloimmune model of streptozotocin induced diabetes. However, unexpectedly we found that Sertoli cells were not consistently extending islet allograft survival in our mouse model and as such we focused on optimizing islet/Sertoli cell co-transplant conditions in the mouse and characterized the difference between successful and unsuccessful islet/Sertoli cell grafts. More specifically we looked at the effect of Sertoli cell amount on islet function and allograft survival in several mouse strains of streptozotocin induced diabetes. Additionally, we assessed both successful and unsuccessful islet/Sertoli cell grafts for islet and Sertoli cell architecture and characterized CD4, CD8 T-cell and macrophage cell infiltrate to see what cells of the immune system Sertoli cells are affecting.

The focus of subsequent chapters was on characterizing the effect of Sertoli cell secreted factors in conditioned media on *in vitro* immune assays to further our understanding of how Sertoli cells inhibit immune function. In Chapter III, mouse Sertoli cell conditioned media was tested for its effect on mitogen (Concanavalin-A) stimulated lymphocyte proliferation. Once T-cells become activated they proliferate and differentiate into effector cells that can respond to invading pathogens or allografts. Rat Sertoli cell conditioned media has been shown to inhibit mitogen stimulated lymphocyte proliferation (226-228). In order to assure our mouse Sertoli cell conditioned media was biologically active we tested it on mitogen stimulated lymphocyte proliferation before testing in other immunological assays. In Chapter IV, mouse Sertoli cell conditioned media was tested for its effect on the CTL, granzyme B, pathway to apoptotic target cell

death. The CTL is a main immune mediator in graft rejection that destroys its target via the delivery of cytolytic molecules such as perforin and granzyme B (230-233). Previous studies have shown that Sertoli cells secrete multiple ligands for the M6P/IGF-II receptor, also found to be a death receptor for granzyme B (256;264). In Chapter IV we found that Sertoli cell conditioned media inhibits granzyme B-mediated apoptosis. We then went on to characterize how Sertoli cell conditioned media inhibits granzyme B killing by studying the effects of Sertoli media on 1) granzyme B receptor expression, 2) granzyme B binding and uptake, and 3) granzyme B enzymatic activity. Sertoli cell conditioned media was found to have no effect on M6P/IGF-II receptor expression, nor was it found to have any effect on granzyme B binding and uptake through its receptor. However, we did find that Sertoli cell conditioned media inhibited granzyme B enzymatic activity through the formation of a stable complex. We suspected that the Sertoli cell secreted factor responsible for the inhibition of granzyme B enzymatic activity was SPI-6, a potent inhibitor of granzyme B known to be expressed by Sertoli cells (259;261;265). We found, through western blot analysis, that this Sertoli cell secreted inhibitor of granzyme B was not SPI-6 but perhaps an unidentified serpin that binds to granzyme B in a fashion similar (irreversibly) to SPI-6.

Finally, in Chapter V we characterized the expression of the M6P/IGF-II receptor for granzyme B in dissociated mouse islets and pancreatic cell lines.

In all, these experiments are aimed at a better understanding of how Sertoli cells interact with immune cells to limit their responsiveness to foreign tissue in an attempt to optimize conditions for islet allograft protection.

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CHAPTER II

COMPARISON OF SUCCESSFUL AND UNSUCCESSFUL GRAFTS OF ALLOGENEIC ISLETS COTRANSPLANTED WITH SERTOLI CELLS

II-A) INTRODUCTION

Islet transplantation using what has come to be known as the Edmonton protocol has been tremendously successful at helping dramatically improve blood glucose control and quality of life in many patients with type 1 diabetes (1-4). However, risks associated with long-term immunosuppression therapy and a shortage of donor organs for transplantation currently limit this procedure to patients with brittle type 1 diabetes (1). One alternative to long-term immune suppression that has been studied experimentally is the co-transplantation of islets with Sertoli cells (5).

Sertoli cells have been called the "nurse cells" of the testes as they aid in the growth and development of new sperm as well as protect sperm from harmful immune destruction (6). Sertoli cells are found closely associated with one another via tight junctions (blood-testis barrier) around the inside of the seminiferous tubules (Figure 1-4). Developing germ cells migrate from the basal edge of the tubule to the luminal edge and are surrounded and supported by Sertoli cells during this process (6). Islet allo- and xenografts have been shown to survive for extended periods of time when transplanted into the interstitial space of the intra-abdominally placed testis of diabetic rats (7;8). Despite successful prolongation of islet graft survival within the testis of rodents, the potential clinical application of these findings is limited.

Many labs have successfully re-created the immune privileged environment of the testis beneath the kidney capsule of diabetic rodents. Sertoli cells have been shown to protect islet allografts (9-12) in diabetic rats and mice, islet xenografts (13;14) in diabetic mice, and islet isografts (15) in non-obese diabetic (NOD) mice from immune rejection. However, syngeneic NOD Sertoli cells have also been shown to result in the destruction of islet isografts in the NOD mouse (15). In that study, doses of 1, 2 and 4 x 10^6 Sertoli cells were found to prolong islet graft survival time; whereas, 8 x 10^6 Sertoli cells was found to decrease islet graft survival time as compared to islet only controls. Graft failure with 8 x 10^6 Sertoli cells was attributed to elevated Fas ligand (FasL) expression by Sertoli cells resulting in neutrophil chemotaxis and graft destruction. Sertoli cell expression of FasL in the alloimmune model, however, has been shown to protect testicular tissue and islets from rejection (11;16). Additionally, in the alloimmune model of chemically-induced diabetes, too few or too many Sertoli cells co-transplanted with islets led to a decrease in islet graft survival time (10-12). For example, our lab has shown previously that co-transplantation of islet allografts with 11 x 10^6 testicular cell aggregates (75% Sertoli cells) beneath the kidney capsule of diabetic rats can prolong islet allograft survival (>95 days in 100% of animals) (10). Similar grafts consisting of islets alone or islets plus 50% fewer testicular cell aggregates survived for only 10 days. Additionally, we have shown in another report in the mouse model that transplantation of islet allografts into sites pre-engrafted with 4.8 x 10^6 Sertoli cells beneath the kidney capsule of diabetic mice can prolong islet allograft survival (66 days) (12). However, transplantation of islet allografts into sites pre-engrafted with more Sertoli cells (6.4 x 10^6) reduced islet allograft survival time (32 days). What has not been addressed in the

alloimmune model is why certain amounts of Sertoli cells lead to successful islet graft prolongation while other amounts are unsuccessful at prolonging graft survival.

Therefore, the main goal of the present study was to characterize why certain amounts of Sertoli cells successfully prolong islet allograft survival while other amounts do not and whether or not Sertoli cells were detrimental to islet graft function in a syngeneic mouse model other than NOD. To do this, we co-transplanted 500 BALB/c islets with 1, 2, 4, or 8 x 10^6 BALB/c Sertoli cells beneath the kidney capsule of streptozotocin induced diabetic BALB/c, C3H-HeJ (C3H-weak responder), and C57BL/6 (B6-strong responder) mice. We subsequently characterized both successful and unsuccessful islet/Sertoli cell grafts for islet and Sertoli cell architecture as well as CD8, and CD4 T-cell and macrophage infiltrate. We observed a correlation between prolonged graft survival and Sertoli cell tubule formation.

II-B) MATERIALS AND METHODS

1. Animals

Male BALB/c (H-2^d) mice (University of Alberta, Edmonton, Alberta, Canada) were used as islet (aged 6-8 weeks) and Sertoli (aged 9-12 days) cell donors as well as transplant recipients (aged 6-8 weeks) of syngeneic islet alone or islet/Sertoli cell grafts. In addition, male C3H (H-2^k) and B6 (H-2^b) mice (Jackson Laboratories, Bar Harbor, Maine), aged 6-8 weeks, were used as transplant recipients of allogeneic islet alone or islet/Sertoli cell grafts. Care and maintenance of all animals was in accordance with guidelines of the Canadian Council of Animal Care.

2. Streptozotocin Administration

All recipients were rendered diabetic with streptozotocin (Sigma, Canada, Oakville, Ontario), a β-cell toxin, at doses of 225mg/Kg (intravenously), 350mg/Kg (intravenously), 200mg/Kg (intraperitoneally), for BALB/c, C3H, and B6 mice respectively, 2-3 days before transplantation. Blood samples were obtained from tail vein bleeds of nonfasting animals. Animals with blood glucose values, measured using a blood glucose meter (One Touch Ultra; LifeScan, Canada), of ≥ 20 mmol/l were used as recipients of islet alone or islet/Sertoli cell grafts.

3. Isolation of Mouse Sertoli Cells

Testicles were isolated from 9-12 day old male BALB/c mouse donors and placed in Hanks' balanced salt solution (HBSS) with 0.5% bovine serum albumin (BSA, Sigma), on ice. Testicles were then chopped in a sterile flow hood with scissors, and digested in a shaking water bath for 6 minutes at 37°C with collagenase (1mg/ml; Sigma Type V) in HBSS. The tissue was then washed three times with HBSS and digested in a siliconized 250ml flask a second time in a shaking water bath for 6 minutes at 37°C with DNase (0.4mg/ml, Boehringer Mannheim, Laval, Canada) and trypsin (1mg/ml, Boehringer) in calcium-free media containing 1mmol/l EGTA and 0.5% BSA (Sigma). Following the second digest the cells were washed with HBSS and filtered through a 500μm nylon mesh, washed an additional three times and resuspended in HAM's F10 culture media supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, and 10% horse serum, and cultured in nontissue culture-coated Petri dishes for 3 days at 37°C and 5% CO_2 before transplantation. These Sertoli cell preparations have been previously

characterized to contain >75% Sertoli cells with the rest being germ cells and peritubular myoid cells (10;12).

4. Isolation of Mouse Islets

Male BALB/c mice (aged 6-8 weeks) were used as islet donors. Access to the pancreas of these donors was gained through a midline incision. The pancreas was then distended with collagenase (1mg/ml, Sigma Type V) in HBSS via the main pancreatic duct, excised, and placed in cold HBSS until all pancreases were harvested for digestion. Pancreases were then transferred to collagenase and digested in a shaking water bath for 13.45 minutes at 37°C. Following digestion with collagenase the tissue was given a quick hand shake to break up any chunks of tissue, and then cold HBSS was added to stop the digestion. Pancreatic tissue was then pelleted, resuspended in fresh HBSS, and filtered through a 500μm mesh. Tissue was then divided into tubes, pelleted, and Ficoll of varying densities (25%, 23%, 21.5%, and 11.5%; bottom to top respectively) was layered over the tissue in preparation for Ficoll density gradient centrifugation. Tissue was then spun at 2000 RPM for 10 minutes with no brake. Following this purification step islets were picked from the first layer of Ficoll and placed into a clean tube with HBSS and washed several times to remove the Ficoll. Islets were then put through a second Ficoll density gradient centrifugation, were picked again from the first layer, washed, and resuspended in M-199 media with 100 U/ml penicillin, 100 U/ml streptomycin (P/S) and 10% newborn calf serum, and hand-picked for the removal of any exocrine tissue, and cultured for 24 hours at 37°C and 5% CO_2 prior to transplantation. These islet preparations have been previously characterized to contain >90% intact islet tissue and <5% exocrine tissue (10).

5. Co-Transplantation of Mouse Islets with Sertoli Cells

Diabetic male BALB/c, C3H, and C57BL/6 mice received 500 hand-picked BALB/c islets alone or in combination with 1, 2, 4, or 8 x 10^6 BALB/c Sertoli cells, with the exception of BALB/c recipients who only received islets in combination with doses of 4 and 8 x 10^6 Sertoli cells. The number of Sertoli cells grafted was assessed using a DNA assay.

Briefly, samples were washed in citrate buffer (150mmol/L NaCl; 15mmol/L citrate; 3mmol/L EDTA; pH 7.4) and stored frozen (-20°C) as dry pellets. Prior to assay, the cell pellets were resuspended in lysis buffer (1000 µl; 10mmol/L Tris; 1mmol/L EDTA; 0.5% Triton X-100; 4°C; pH 7.5), and sonicated. Aliquots were assayed in duplicate by diluting them in Tris-EDTA (1ml; 10mmol/L, 1mmol/L EDTA; pH 7.5) and measuring florescence (490 excitation and emission filter wavelength of 515 nm) after the addition of Pico Green double stranded DNA reagent (1ml; 1:200 dilution with DNA buffer; Molecular Probes, Eugene, Oregon). Samples were run in parallel with and diluted in proportion to standards of known DNA content for sample DNA content determination. Calculations were based on our observation that freshly isolated testicular cells contained ~20 pg DNA/cell.

Prior to transplantation a known number of Sertoli cells were mixed together with 500 islets in polypropylene microcentrifuge tubes. Grafts were aspirated into polyethylene tubing (PE-50), pelleted by centrifugation, and placed beneath the left kidney capsule of halothane-anesthetized recipients. Recipients were allowed to recover and blood glucose was monitored three times a week to track the success of the graft. Diabetes or hyperglycemia was considered corrected by the islet graft if blood glucose

was found to be lowered to between 4 and 7 mmol/L. Graft bearing kidneys were harvested at the time of rejection (return to hyperglycemia-graft failure, two consecutive readings of ≥ 18 mmol/L) or at the end of the study (>60 days) if mice remained normoglycemic (successful graft, blood glucose between 4 and 7 mmol/L) for immunohistochemical analysis and to confirm that normal blood glucose was dependent on the islet graft.

6. Oral Glucose Tolerance Test

Oral glucose tolerance testing was performed on male BALB/c mouse recipients of 500 BALB/c islets alone or in combination with 4 or 8 x 10^6 BALB/c Sertoli cells on day 60 post transplantation in order to determine if Sertoli cells have an effect on islet graft function. Dextrose (3mg/g body weight) was administered by oral gavage into mice fasted for 24 hours. Blood glucose levels were measured at times 0, 15, 30, 60, and 120 minutes following dextrose administration.

7. Immunohistochemical Analysis

All grafts were cut in half with half of the graft immersed in Z-fix (Davis Diagnostics Inc., Mississauga, Ontario), embedded in paraffin, sectioned, and stained for insulin (islet β-cells), hematoxylin-eosin (H/E; cellular infiltrate), and GATA-4 (Sertoli cells) and the other half placed in a cartridge with cryomatrix (Fisher Scientific, Ottawa, Ontario), frozen and later sectioned, and stained for CD4, and CD8 T-cells, as well as macrophages.

For insulin staining, slides were incubated for 6 minutes with 10% hydrogen peroxide in methanol to quench endogenous peroxidases, blocked for 15 minutes with 20% normal goat serum, and incubated for 30 minutes with guinea pig anti-insulin

(1:1000; Dako, Carpinteria, California). Slides were then incubated for 20 minutes with biotinylated goat anti-guinea pig antibody (1:200; Vector, Burlingame, California) followed by peroxidase-streptavidin with diaminobenzadine (DAB) as the chromagen (brown stain). Finally, slides were counterstained with hematoxylin-eosin.

For GATA-4 staining, slides were heated in a microwave for 15 minutes in 0.01M sodium citrate buffer (pH 6.0), incubated with 10% hydrogen peroxide in methanol, blocked with 20% normal rabbit serum, and incubated with goat anti-GATA-4 (1:25 prepared in 20% normal rabbit serum; Santa Cruz Biotechnology, Santa Cruz, California). Slides were then incubated for 20 minutes with biotinylated horse anti-goat antibody (1:200; Vector) followed by peroxidase-streptavidin, DAB and counterstaining with Harris's hematoxylin.

For CD4, and CD8 T-cells, as well as macrophage staining, slides were fixed for 3 minutes in acetone at -20°C, and blocked for 20 minutes in 2% fetal bovine serum (FBS) in phosphate buffered saline (PBS) followed by additional blocking with avidin and biotin blocking solution (Vector). Slides were then incubated for 30 minutes with rat anti-CD4 (1:100), -CD8 (1:100), and -CD11b (1:500, macrophage marker) (Becton Dickinson Pharmingen, San Jose, California) followed by a 30 minute incubation with biotinylated anti-rat antibody (1:200, Vector). Slides were then incubated with peroxidase-streptavidin, DAB and counterstained with Harris's hematoxylin.

8. Statistical Analysis

Data is expressed as the mean ± standard error of the mean (SEM) of *n* independent experiments and p-values are listed as compared to the islet only controls. Blood glucose level determination was considered a dependent measure because multiple

samples were taken over time from each mouse; therefore, requiring a repeated measures analysis of variance (ANOVA) for the determination of statistical significance of differences between groups. A Kaplan-Meier was used to plot a survival curve and a log rank test was used to assess statistical significance of differences between groups for graft survival. A value of $p < 0.05$ was considered significant.

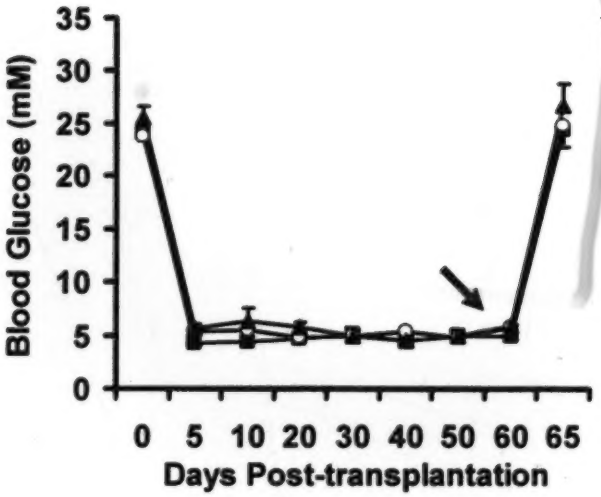
II-C) RESULTS

1. The Effect of Sertoli Cells on Islet Graft Function

To investigate the effect of Sertoli cells on islet graft function, 500 BALB/c islets were transplanted alone (n=10) or in combination with 4 (n=10) or 8 x 10^6 (n=6) BALB/c Sertoli cells beneath the kidney capsule of streptozotocin induced diabetic BALB/c mice (syngeneic transplant). All recipient animals became normoglycemic within three days of receiving their transplant and maintained excellent blood glucose control throughout the follow up period regardless of the amount of Sertoli cells co-transplanted (p=0.066 versus control, Figure 2-1A). Oral glucose tolerance tests performed on day 60 post-transplantation showed no significant difference between groups in their ability to recover from a glucose challenge (p=0.608 versus control, Figure 2-1B). Animals in all groups quickly returned to their original hyperglycemic state upon removal of the graft bearing kidney indicating that the graft was responsible for the maintenance of normoglycemia in recipient mice (Figure 2-1A, arrow). Histological examination of grafts stained for GATA-4 (Sertoli cell marker) revealed Sertoli cells arranged in tubule-like structures with their nuclei arranged around the outer edge of the tubules (Figure 2-2A) in a manner similar to how Sertoli cells appear in their native testis in the

seminiferous tubules (Figure 2-2B). Larger numbers of Sertoli cells (8×10^6) or prolongation of the post-transplantation period (100 days) resulted in even larger Sertoli tubule structures. Histological examination of grafts stained for insulin (islet marker) revealed islets surrounding large Sertoli cell tubule structures and appearing both fragmented and intact (Figure 2-2C). When islets were transplanted alone they appeared intact (Figure 2-2D). Islets appeared even more fragmented when co-transplanted with larger numbers of Sertoli cells (8×10^6) or if the post-transplantation period was extended (100 days). However, despite islet fragmentation in the presence of Sertoli cells no deleterious effect of Sertoli cells on islet graft function was found. No significant amount of cellular infiltrate was observed in islet grafts regardless of Sertoli cell co-transplant number.

A



B

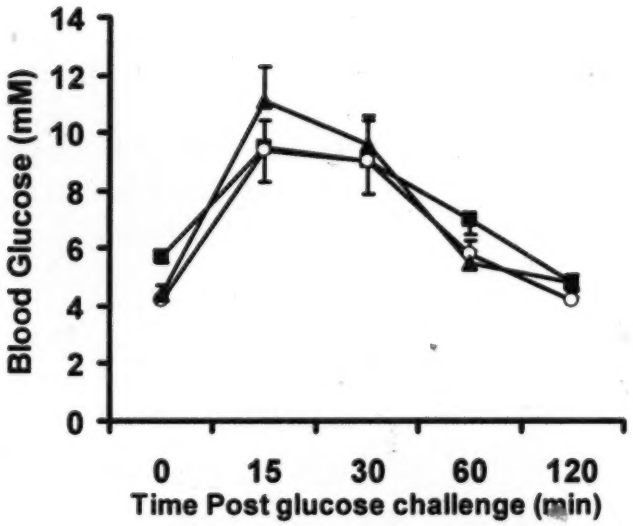


Figure 2-1. Co-transplantation of islets with Sertoli cells has no deleterious effect on islet graft function. (A) Average blood glucose values ± SEM of BALB/c mice up to 60 days post-transplantation with 500 BALB/c islets alone (solid squares; n=10), or in combination with 4 (open circles; n=10) or 8 x 10^6 (solid triangles; n=6) BALB/c Sertoli cells. All animals returned to hyperglycemia following the removal of the graft-bearing kidney (arrow). (B) Oral glucose tolerance test performed on the same groups of transplant recipients on day 60 post-transplantation. Blood glucose values are presented as the mean ± SEM.

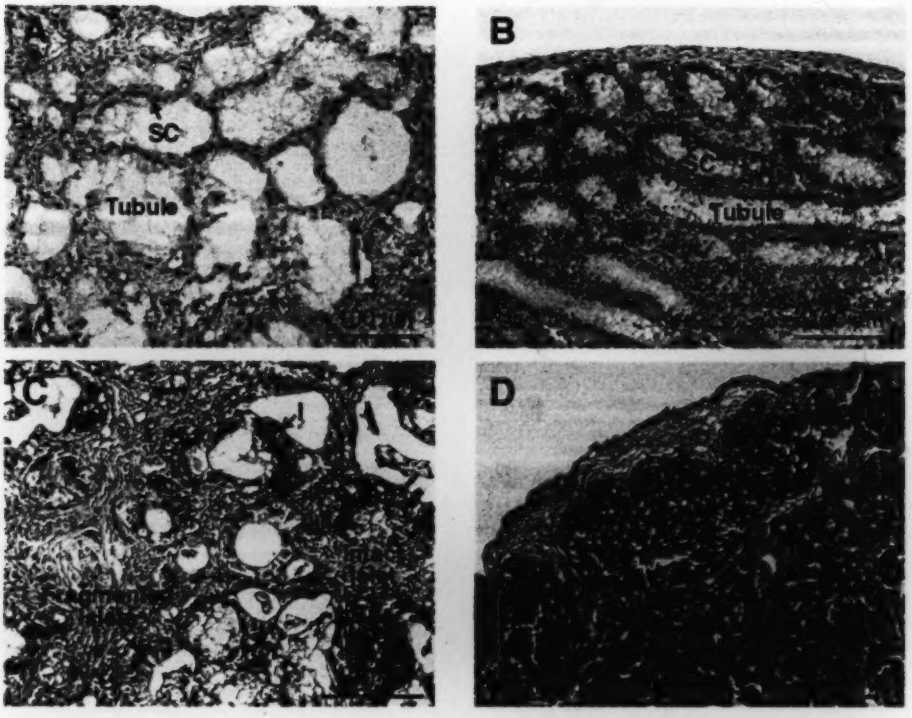


Figure 2-2. Sertoli cells form tubule-like structures post co-transplantation with islets in a syngeneic mouse model. 500 BALB/c islets were transplanted alone or in combination with 4 or 8 x 10^6 BALB/c Sertoli cells beneath the kidney capsule of diabetic BALB/c mice. Grafts were harvested from normoglycemic animals on day 60 post transplantation for immunohistochemical analysis. GATA-4 staining for the localization of Sertoli cells (SC, brown) in (A) islet/Sertoli cell (8 x 10^6) grafts and (B) the mouse testis. Sertoli cells form tubule-like structures, similar to how they are arranged in their native testis, post co-transplantation with islets in the BALB/c mouse. Insulin and H/E staining for the localization of islets (I, brown) and cellular infiltrate, respectively, in (C) islet/Sertoli cell (4 x 10^6) grafts and (D) islet only grafts. Islets surround Sertoli tubule structures and appear either fragmented or intact (as in islet only grafts) post co-transplantation with Sertoli cells in the BALB/c mouse.

2. Prolongation of Islet Allograft Survival by Co-Transplantation with Sertoli Cells

To examine the ability of Sertoli cells to prolong islet allograft survival and to discover the optimal amount of Sertoli cells for co-transplantation with islets, 500 BALB/c islets were transplanted alone or in combination with 1, 2, 4, or 8 x 10^6 BALB/c Sertoli cells beneath the kidney capsule of streptozotocin induced diabetic C3H or B6 mouse recipients.

C3H mouse recipients of islet grafts alone or in combination with 1, 2, 4, or 8 x 10^6 Sertoli cells all became normoglycemic within 3 days of receiving their graft and all animals returned to hyperglycemia following the removal of their graft bearing kidney in cases of long term (>60 days) graft survival. Blood glucose control was excellent (between 4 and 7 mmol/L) in all animals with long term graft survival in the C3H mouse. Unexpectedly, 5 out of 23 islet alone grafts survived for more than 60 days in diabetic C3H recipients (Table 2-1). Co-transplantation of islets with 1 x 10^6 Sertoli cells did not significantly ($p=0.070$ versus control) prolong islet graft survival time in the C3H mouse with only 1 out of 12 grafts (8.3%) surviving beyond 60 days. Co-transplantation of islets with 2 x 10^6 Sertoli cells did prolong 4 out of 10 grafts (40%) beyond 60 days; however, overall graft prolongation was not found to be significant ($p=0.628$). Co-transplantation of islets with 4 x 10^6 Sertoli cells was found to be the optimum amount in the C3H mouse as islet graft survival was significantly ($p=0.005$) prolonged using this amount of Sertoli cells, with 8 out of 13 grafts (61.5%) surviving beyond 60 days. However, co-transplantation of islets with 8 x 10^6 Sertoli cells was found to be too many as there was no significant ($p=0.731$) prolongation of graft survival time, with only 3 out of 11 grafts (27.3%) surviving beyond 60 days.

Because the C3H mouse was found to be a so-called "weak responder" to the BALB/c islet allograft we decided to examine the ability of Sertoli cells to prolong BALB/c islet allograft survival in the B6 mouse, known to be a "strong responder" to islet allografts (17;18).

B6 mouse recipients of islet grafts alone or in combination with 1, 2, 4, or 8 x 10^6 Sertoli cells all became normoglycemic within 3 days of receiving their graft, with the exception of 2 out of 10 mice that had primary non function of islets co-transplanted with 8 x 10^6 Sertoli cells. All animals returned to hyperglycemia following the removal of their graft bearing kidney in cases of long term (>60 days) graft survival. Blood glucose control was poor (fluctuated above 10 mmol/L) in all animals with long term graft survival in the B6 mouse. BALB/c islets alone survived for only 13 days in 8 out of 14 B6 recipients, proving that the B6 mouse is a much stronger responder to the BALB/c islet allograft (Table 2-2). Co-transplantation of islets with 1 x 10^6 Sertoli cells was found to significantly ($p=0.031$) prolong islet allograft survival, with 2 out of 10 grafts (20%) surviving beyond 60 days and one graft still ongoing (>41 days). Co-transplantation of islets with 2 x 10^6 Sertoli cells also significantly ($p=0.018$) prolonged islet allograft survival in the B6 mouse with no grafts (0%) surviving beyond 60 days to date but 2 grafts are still ongoing (>34, and >41 days). Co-transplantation of islets with 4 x 10^6 Sertoli cells modestly improved graft survival time with 1 out of 12 grafts (8.3%) surviving beyond 60 days (>100); however, there was no significant ($p=0.058$) overall prolongation of graft survival time with this amount of Sertoli cells. Again, co-transplantation of islets with 8 x 10^6 Sertoli cells was found to be too many with no

significant (p=0.681) prolongation of islet allograft survival time and no islet grafts (0%) surviving beyond 60 days.

Table 2-1. Survival of 500 BALB/c islets co-transplanted with various amounts of BALB/c Sertoli cells beneath the kidney capsule of diabetic C3H recipients.

Sertoli Cells ($x10^6$)	Graft Survival (Days)	% Survival Past 60 Days
none	19,24,25,26,27,28,31,32,34,41,41,41 41,41,45,45,48,49,65,>60,>60,>68,>84	21.7%
1	16,17,20,23,23,26,30,32,32,43,47,>84	8.3%
2	19,20,23,41,41,42,61,68,>81,>81	40.0%
4*	21,42,45,48,55,>68,>68,>73,>74,>80,>80,>80,>87	61.5%
8	16,22,23,25,31,32,38,40,>68,>80,>91	27.3%

>Graft-bearing kidneys were harvested from normoglycemic animals on days indicated to confirm return to hyperglycemia and for immunohistochemical analysis. *Graft survival was significantly prolonged ($p<0.05$) as compared to the control with islets alone.

Table 2-2. Survival of 500 BALB/c islets co-transplanted with various amounts of BALB/c Sertoli cells beneath the kidney capsule of diabetic B6 recipients.

Sertoli Cells ($x10^6$)	Graft Survival (Days)	% Survival Past 60 Days
none	7,11,13,13,13,13,13,13,13,13,16,18,24,>59	7.1%
1*	13,15,16,18,18,18,19,>41,>100,>100	20.0%
2*	14,18,18,18,20,28,29,>34,>41	0
4	13,13,17,17,18,21,21,24,25,26,47,>100	8.3%
8	12,12,14,16,16,18,20,36	0

>Graft-bearing kidneys were harvested from normoglycemic animals on days indicated to confirm return to hyperglycemia and for immunohistochemical analysis. *Graft survival was significantly prolonged ($p<0.05$) as compared to the control with islets alone.

3. Histological Examination of Successful and Unsuccessful Islet/Sertoli Cell Allografts

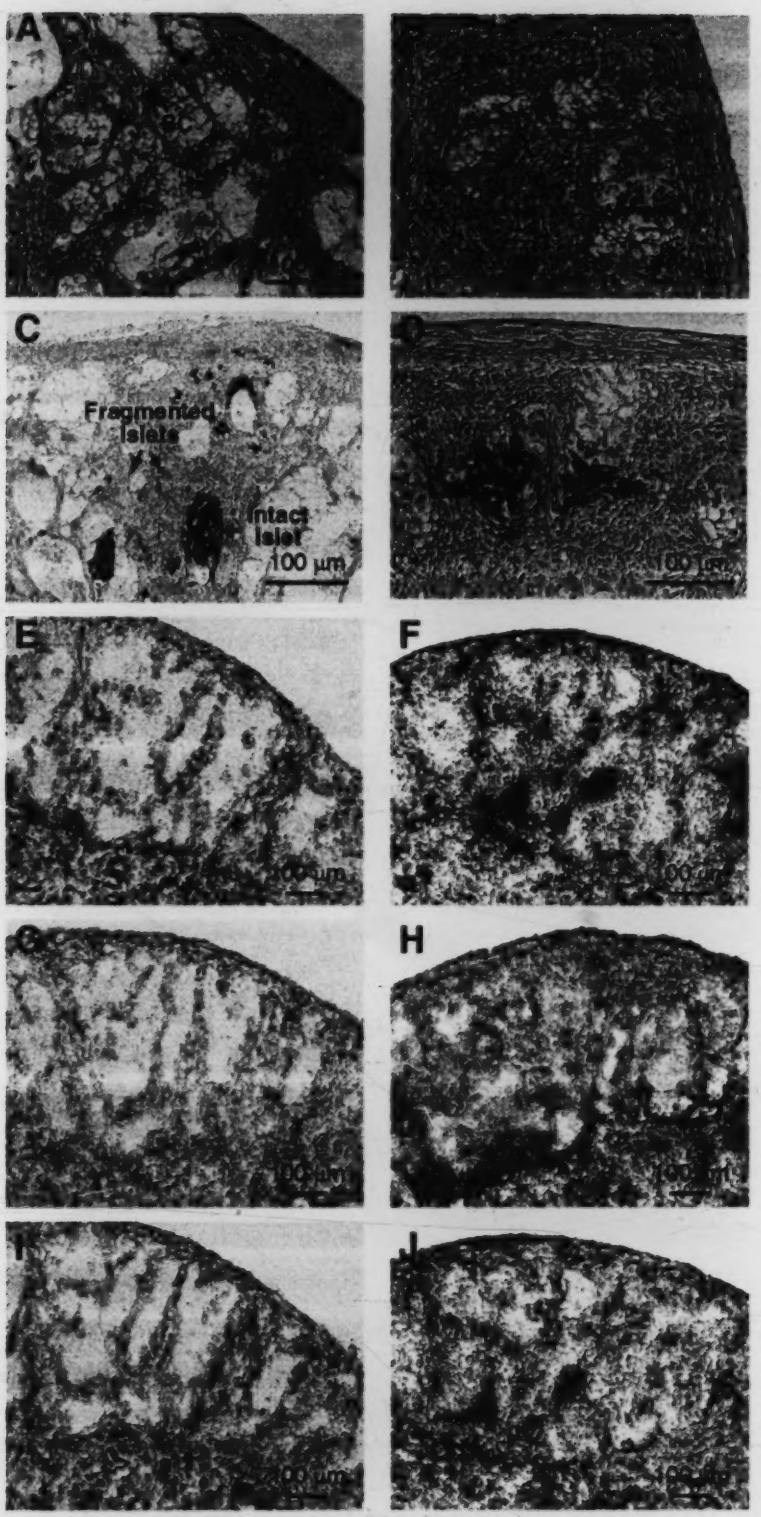
In order to understand the difference between successful and unsuccessful islet/Sertoli cell grafts, defined as extended graft survival beyond 60 days or graft failure before 60 days, respectively, we performed immunohistochemical analysis on grafts harvested from C3H and B6 mice. Grafts were stained for the detection of Sertoli cells, islets, CD4, and CD8 T cells, as well as macrophages.

Examination of successful grafts (islets + 4 x 10^6 Sertoli cells) harvested from normoglycemic C3H mice on day 80 post-transplantation revealed Sertoli cells arranged in large tubule-like structures with their nuclei around the outer edge of the tubules (Figure 2-3A). In comparison, Sertoli cells in unsuccessful grafts (islets + 2 x 10^6 Sertoli cells) harvested from hyperglycemic C3H mice were found to be arranged in aggregates with nuclei scattered throughout the Sertoli cell aggregates (Figure 2-3B). Islets in both successful (Figure 2-3C) and unsuccessful (Figure 2-3D) grafts with Sertoli cells appeared both intact and fragmented with islets surrounding Sertoli cell tubules and aggregates, respectively. A minimal amount of CD4 T cells (Figure 2-3E) and macrophages (Figure 2-3G) were found throughout successful islet/Sertoli cell grafts harvested from C3H mice; however, strikingly, CD8 T cells (Figure 2-3I) were absent. In comparison, marked CD4 T cells (Figure 2-3F), macrophage (Figure 2-3H), and CD8 T cells (Figure 2-3J) were observed in unsuccessful islet/Sertoli cell grafts.

Examination of successful grafts (islets + 1 x 10^6 Sertoli cells) harvested from normoglycemic B6 mice on day 100 post-transplantation revealed Sertoli cells arranged in aggregates and small tubule-like structures (Figure 2-4A). In comparison, Sertoli cells in unsuccessful grafts (islets + 4 x 10^6 Sertoli cells) harvested from hyperglycemic B6

mice were found to be arranged randomly and in aggregates (Figure 2-4B). Islets in both successful (Figure 2-4C) and unsuccessful (Figure 2-4D) grafts with Sertoli cells appeared mainly fragmented with islets surrounding Sertoli cell tubule and aggregate formations. CD4 T cells (Figure 2-4E), macrophages (Figure 2-4G), and CD8 T cells (Figure 2-4I) were found throughout successful islet/Sertoli cell grafts harvested from B6 mice; CD4 T cell and macrophage infiltrate was striking, however, CD8 T cell infiltrate was minimal. In comparison, marked CD4 T cells (Figure 2-4F), macrophages (Figure 2-4H), and CD8 T cells (Figure 2-4J) were observed in unsuccessful islet/Sertoli cell grafts. Notably, in unsuccessful grafts, where no islets were found, Sertoli cells were seen in abundance.

Figure 2-3. Histological examination of successful versus unsuccessful islet/Sertoli cell allografts harvested from C3H mice. The representative successful graft shown here (column 1) was harvested from a normoglycemic C3H mouse on day 80 post-transplantation with 500 BALB/c islets and 4 x 10^6 BALB/c Sertoli cells. The representative unsuccessful graft shown here (column 2) was harvested from a hyperglycemic C3H mouse on day 41 post-transplantation with 500 BALB/c islets and 2 x 10^6 BALB/c Sertoli cells. (A, B) GATA-4 staining for the localization of Sertoli cells (SC, brown). (C, D) Insulin staining for the localization of islets (I, brown). (E, F) CD4 staining for the localization of CD4 T-cells (brown). (G, H) CD11b staining for the localization of macrophages (brown). (I, J) CD8 staining for the localization of CD8 T-cells (brown).


A
C
Fragmented Islets
Intact Islet
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I
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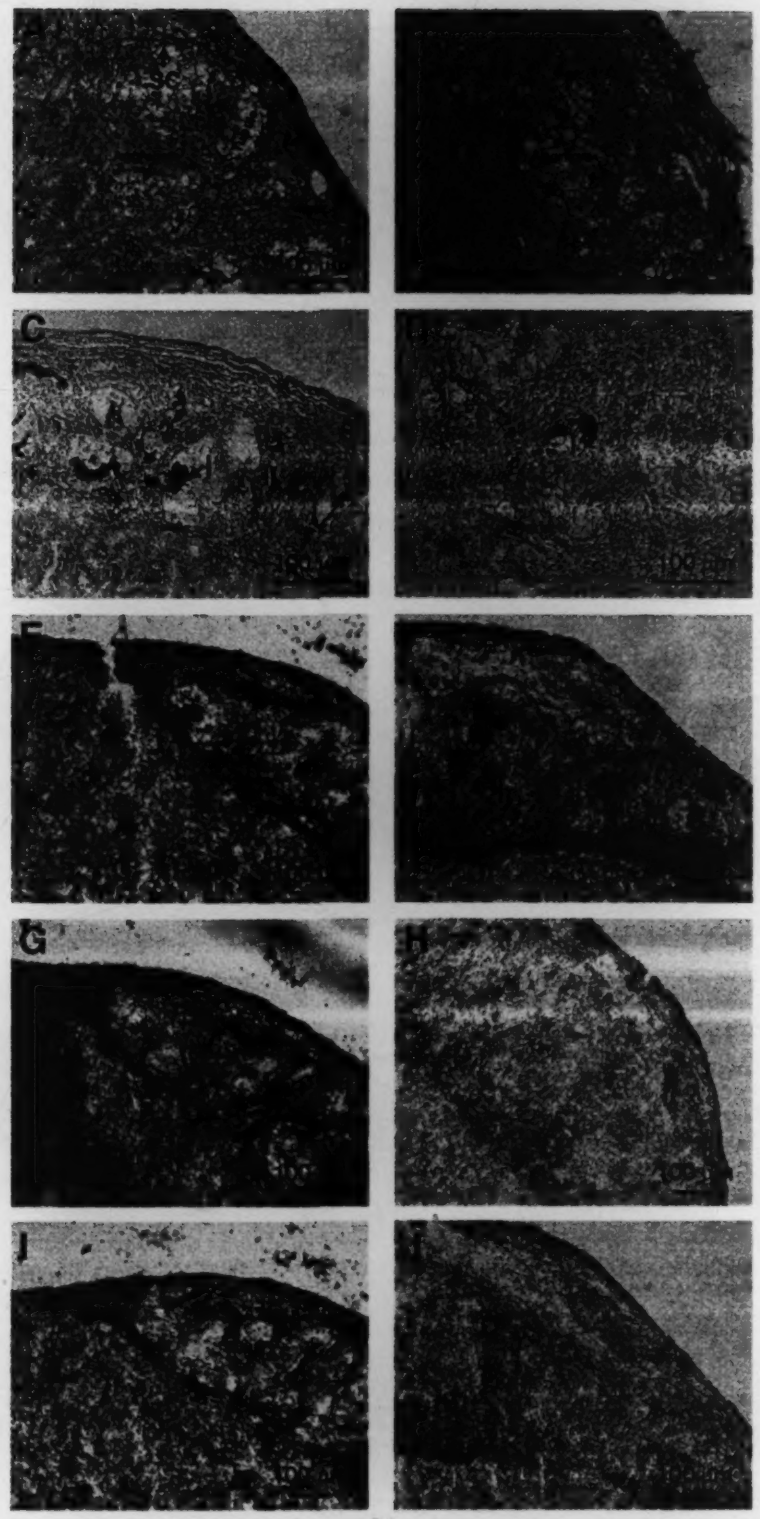
Figure 2-4. Histological examination of successful versus unsuccessful islet/Sertoli cell allografts harvested from B6 mice. The representative successful graft shown here (column 1) was harvested from a normoglycemic B6 mouse on day 100 post-transplantation with 500 BALB/c islets and 1 x 10^6 BALB/c Sertoli cells. The representative unsuccessful graft shown here (column 2) was harvested from a hyperglycemic B6 mouse on day 24 post-transplantation with 500 BALB/c islets and 4 x 10^6 BALB/c Sertoli cells. (A, B) GATA-4 staining for the localization of Sertoli cells (SC, brown). (C, D) Insulin staining for the localization of islets (I, brown). (E, F) CD4 staining for the localization of CD4 T-cells (brown). (G, H) CD11b staining for the localization of macrophages (brown). (I, J) CD8 staining for the localization of CD8 T-cells (brown).


A
C
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II-D) DISCUSSION

In the present study we found that co-transplantation of 500 BALB/c islets with 4 or 8 x 10^6 BALB/c Sertoli cells beneath the kidney capsule of streptozotocin induced diabetic BALB/c mice had no deleterious effect on islet graft function. Similarly, in another syngeneic model of streptozotocin induced diabetes, Kin *et al.* found that 5.7 x 10^6 BALB/c Sertoli cells preengrafted beneath the kidney capsule of BALB/c mice had no adverse effect on BALB/c islet graft function (12). However, in contrast, Korbutt *et al.* found that co-transplantation of 500 NOD islets with 8 x 10^6 NOD Sertoli cells beneath the kidney capsule of spontaneously diabetic NOD mice resulted in rapid graft destruction by neutrophils, attributed to Sertoli cell expression of FasL (15). Therefore, it appears that co-transplantation of islets with higher numbers of syngeneic Sertoli cells is detrimental to islet graft function in the NOD mouse but not in the BALB/c mouse. The reason for this contradiction is uncertain. Potentially the NOD background makes the NOD mouse more susceptible to inflammation as compared to the BALB/c mouse.

Our lab has shown previously that co-transplantation of islet allografts with Sertoli cells beneath the kidney capsule of diabetic rats can prolong islet allograft survival (>95 days in 100% of animals) (10). The original goal of this Chapter was to reproduce these findings in a mouse model in order to examine the mechanism of how Sertoli cells might be protecting islet allografts. The choice of mouse over rat was made due to the availability of mouse monoclonal antibodies and knockout mice that could be used in the examination of the mechanism of Sertoli cell protection. For example, transforming growth factor-beta (TGF-β) has been implicated in the mechanism of how Sertoli cells protect islets from autoimmune destruction in the NOD mouse model using

monoclonal antibodies directed at TGF-β (19). We were interested in examining if this is also the case in the alloimmune model of streptozotocin induced diabetes. However, unexpectedly, we found that Sertoli cells did not consistently extending islet allograft survival in our mouse models. Our focus then turned to finding an optimum number of Sertoli cells for co-transplantation with islets for maximum extension of islet allograft survival. Additionally, we focused on characterizing the difference between successful and unsuccessful islet/Sertoli cell grafts.

We found that co-transplantation of 500 BALB/c islets with 4 x 10^6 BALB/c Sertoli cells was the best combination for prolonging islet allograft survival in the C3H mouse with 8/13 animals remaining normoglycemic beyond 60 days. Co-transplantation of islets with fewer (1 or 2 x 10^6) or more (8 x 10^6) Sertoli cells did not significantly extend islet allograft survival in the C3H mouse. Similarly, Kin *et al.* found that 4.8 x 10^6 BALB/c Sertoli cells, preengrafted beneath the kidney capsule of BALB/c mice, prolonged CBA/J islet allografts survival in the BALB/c mouse (66 days) (12). However, with a larger number (6.4 x 10^6) of Sertoli cells, preengrafted in a similar manner, islet allograft survival time was reduced (32 days). Korbutt *et al.* also found that co-transplantation of islets with an optimal number (11 x 10^6) of Sertoli cells significantly extended islet allograft survival time in the rat (>95 days) (10). Similar grafts consisting of islets with 50% fewer Sertoli cells resulted in reduced survival time to only 10 days. The findings in our present study confirm previous observations and suggest that an optimal amount of Sertoli cells can significantly prolong islet allograft survival in both the rat and mouse models of chemically induced diabetes, with too few or too many Sertoli cells resulting in a reduction in allograft survival time.

Unexpectedly, we found that BALB/c islet allografts transplanted alone (no Sertoli cells) survived for extended periods in the C3H recipient (5/23, >60 days), indicating that the C3H mouse was a weak responder to BALB/c islet allografts. Therefore, we wanted to see if Sertoli cells could extend islet allograft survival in the B6 mouse, known to be a strong responder to allografts (17;18). In the present study we found that BALB/c islets survived on average 13 days in the B6 recipient, proving that the B6 mouse is a strong responder to islet allografts. We found that co-transplantation of 500 BALB/c islets with 1 or 2 x 10^6 BALB/c Sertoli cells was the best combination for prolonging islet allograft survival in the B6 mouse. Co-transplantation of islets with more (4 or 8 x 10^6) Sertoli cells did not significantly extend islet allograft survival. Additionally, 2 out of 10 mice transplanted with islets and 8 x 10^6 Sertoli cells were primary non-function. Takeda *et al.* found that co-transplantation of C3H islets with 4 x 10^5 C3H Sertoli cells was the best combination for prolonging islet allograft survival in the B6 mouse (18 days) (11). Transplantation of islets with fewer (2 x 10^5) or more (2 x 10^6) Sertoli cells did not prolong islet graft survival (10 days) and in the case of the larger Sertoli mass, mice failed to achieve normoglycemia. Even though in our case (BALB/c to B6) 1 and 2 x 10^6 Sertoli cells was found to be the optimal number of Sertoli cells for co-transplantation with islets in the B6 mouse, it is clear that a lower number of Sertoli cells is required in this mouse model to achieve prolonged graft survival; however, prolongation in this model was modest at best. These findings indicate two things: 1) the optimal number of Sertoli cells will have to be determined on a case by case basis (depending on the donor/recipient combination) and 2) Sertoli cells are not as efficient at

significantly extending islet allograft survival in the B6 mouse, a strong responder to islet allografts.

Subsequently, we wanted to understand why the protective nature of Sertoli cells was dependent on 1) Sertoli cell number, and 2) recipient mouse strain (recipient immune response). Histological examination of successful islet/Sertoli cell grafts harvested from normoglycemic C3H mice on day 80 post transplantation revealed Sertoli cells arranged in large tubule-like structures with islets broken and surrounding the tubules. Small tubules were also seen in successful islet/Sertoli cell grafts harvested from the B6 mouse. Similar examination of unsuccessful islet/Sertoli cell grafts harvested from hyperglycemic C3H and B6 mice revealed Sertoli cells arranged either randomly or in aggregates. The tubule arrangement seen in successful grafts closely resembles how Sertoli cells are arranged in their native testis, in the seminiferous tubules (6). In transplant models, neonatal porcine Sertoli cells have also been shown to form tubule-like structures following transplantation into immune compromised SCID mice (20;21). Additionally, syngeneic rat and mouse Sertoli cells have been found to form tubule-like structures in immune competent animal models (12;20). However, this is the first time that Sertoli cells have been shown to form tubule-like structures upon co-transplantation with islets. This is also the first time a correlation has been seen between Sertoli tubule formation and successful graft prolongation. This may explain why successful Sertoli-mediated protection of islet allografts is dependent on both Sertoli number and recipient mouse strain. For example, too few Sertoli cells may not be enough for tubule formation and too many may lead to hypoxic and nutrient-deprived conditions resulting in necrosis and inflammation and subsequent loss of Sertoli cell mass, also resulting in poor tubule

formation. Furthermore, strong immune responses to allografts may prevent Sertoli tubule formation, as we found to be the case in the B6 mouse.

Although Sertoli tubule formation may be a requirement for optimal islet allograft protection, tubule formation may not be required for Sertoli cells to protect themselves from immune destruction. We found that in cases where islets were completely rejected, Sertoli cells were still present in random or aggregate formation.

Besides our current finding that Sertoli cell number and recipient immune response are important for tubule formation and prolonged islet allograft survival there may be additional requirements that need to be examined. For example, our Sertoli cell preparation has been previously characterized to also contain peritubular myoid cells and germ cells (10;12). These other cell types may be required for tubule formation. Another potential requirement for Sertoli cell tubule formation may be tissue culture of Sertoli cells prior to transplantation. Cultured Sertoli cells have been shown to form aggregates, which may be important for tubule formation post-transplantation (10). These and other requirements for Sertoli tubule formation and promotion of extended islet allograft survival should be the focus of future studies.

The benefit of Sertoli arrangement in tubule formation is unknown but it may be that Sertoli cells in this formation can easily communicate with one another via tight junctions to coordinate the secretion of immunosuppressive factors towards islets located outside the tubules. Even though Sertoli cells are known to protect developing germ cells in the seminiferous tubules, the immune privileged site of the testis has been found to extend beyond the seminiferous tubules, into the interstitial space, where islet allo- and xenografts have been shown to survive for extended periods of time (6-8). These

findings would seem to suggest that a Sertoli cell secreted factor(s) may be involved in the protection of bystander islet allografts from immune destruction. The identification of Sertoli cell secreted factors involved in protection of islet allografts have not yet been elucidated but will be the focus of future chapters in this thesis.

Finally, we characterized CD8 and CD4 T-cell, as well as macrophage infiltrate in successful and unsuccessful islet/Sertoli cell grafts. Characterization of successful grafts harvested from both C3H and B6 mice revealed the presence of CD4 T-cells and macrophages but very few CD8 T-cells. Conversely, unsuccessful grafts had marked CD4 and CD8 T-cell, as well as macrophage infiltrate. These findings suggest that co-transplantation of islets with an optimal number of Sertoli cells can protect islet allografts from CD8 T-cell-mediated rejection but may not be able to protect from CD4 T-cell and macrophage-mediated rejection. Future experiments should aim at extending the post-transplantation follow up period to see if these CD4 T-cells and macrophages eventually do end up destroying the graft. Alternatively, these CD4 T-cells could be suppressor T-cells which could be easily identified in the future using immunohistochemistry.

In conclusion, we have demonstrated that Sertoli tubule-formation post co-transplantation with islets may be a requirement for successful prolongation of islet allograft survival. Co-transplantation of islets with too few or too many Sertoli cells may interfere with tubule-formation and result in reduced allograft survival time. Likewise, the presence of an aggressive recipient immune response may also impede tubule-formation. Future studies should test the ability of transient immunosuppression, delivered immediately post co-transplantation of islets with an optimal number of Sertoli cells, to permit Sertoli tubule formation and extended islet allograft survival in the B6

mouse. Optimization of islet/Sertoli cell co-transplantation conditions will help this technology progress to hopefully be a viable alternative to chronic immunosuppressive therapy for the use in clinical islet transplantation.

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CHAPTER III

MOUSE SERTOLI CELL CONDITIONED MEDIA EXERTS BOTH INHIBITORY AND STIMULATORY EFFECTS ON CONCANAVALIN-A-STIMULATED LYMPHOCYTE PROLIFERATION

III-A) INTRODUCTION

One of the major obstacles currently facing islet transplantation for the treatment of type 1 diabetes is the requirement for chronic immunosuppressive therapy (1). Immunosuppression is required to prevent islet graft immune destruction through autoimmune diabetes recurrence and allograft rejection. Islet allografts have been shown to survive for extended periods in the intra-abdominally placed testis of non-immunosuppressed diabetic rats (2). The Sertoli cell has been found to be responsible for the protection of allografts placed within the testis and isolated Sertoli cells, co-transplanted with islets, have additionally been reported to protect islet allografts in diabetic rats and mice, and islet isografts in non-obese diabetic (NOD) mice from immune rejection (3-7). However, what remains to be determined is how Sertoli cells protect islets from immune destruction.

Sertoli cells are closely associated with one another via tight junctions in the seminferous tubules of the testis (8). This arrangement of Sertoli cells creates the blood-testis barrier and helps protect developing germ cells in the seminiferous tubules from harmful immune reactions. However, the immune privileged environment of the testis has been found to extend beyond the seminiferous tubule to include the interstitial space

where islet allografts have been shown to be protected (2). This suggests that a Sertoli cell secreted factor(s) may be responsible for Sertoli cell-mediated immune protection.

Sertoli cells secrete many proteins (Table 1-1), including a variety of growth and immunoregulatory factors (9). Some examples include: seminiferous growth factor, Sertoli cell-secreted growth factor, nerve growth factor, transforming growth factor-alpha and -beta (TGF-α and TGF-β), activin, inhibin, and insulin-like growth factor-I (IGF-I).

Activation of T-cells by both signal 1 (T-cell receptor interaction with peptide-MHC complex) and signal 2 (co-stimulation) up-regulates the expression of IL-2 and IL-2 receptor, leading to proliferation and differentiation of T-cells (lymphocytes) into memory and effector cells capable of destroying the invading pathogen or allograft (10). Mitogens, are agents capable of inducing lymphocyte proliferation (polyclonal activators) (11). Concanavalin-A, for example, is a lectin that stimulates T-cells through binding glycoproteins on the surface of these cells.

Sertoli cell conditioned media, prepared from cultured rat Sertoli cells, has been demonstrated to suppress mitogen stimulated lymphocyte proliferation (12-14). Many Sertoli cell secreted proteins, including TGF-β, activin, inhibin, and IGF-I have been shown to have similar effects on lymphocyte proliferation (15-17). Sertoli cell secreted TGF-β, in particular, has been shown to protect islets from autoimmune destruction in the NOD mouse (18). However, the factor(s) in Sertoli cell conditioned media, with suppressive effects on mitogen stimulated lymphocyte proliferation, has never been characterized; nor has this conditioned media ever been tested for its ability to inhibit other immune mechanisms. Cytotoxic T lymphocytes, for example, are a main immune mediator in allograft rejection (19-22).

Therefore, the main goal of the present study was to prepare Sertoli cell conditioned media, from cultured mouse Sertoli cells, and to test its biological activity, as assessed by its ability to inhibit mitogen (Concanavalin-A) stimulated lymphocyte proliferation, with TGF-β used as an internal control. Once prepared and tested, Sertoli cell conditioned media will be used in future experiments to test its ability to inhibit immune effector responses, such as cytotoxic T lymphocyte (eg. granzyme B) killing, in order to further our understanding of how Sertoli cells interact with the immune system to prevent graft rejection.

III-B) MATERIALS AND METHODS

1. Animals

Male BALB/c (H-2^d) mice (University of Alberta, Edmonton, Alberta, Canada) were used as Sertoli (aged 9-12 days) cell donors. In addition, male C57BL/6 (B6, H-2^b) mice (Jackson Laboratories, Bar Harbor, Maine), aged 6-8 weeks, were used as lymph node donors for the isolation of lymphocyte responder cells. Care and maintenance of all animals was in accordance with guidelines of the Canadian Council of Animal Care.

2. Isolation of Mouse Sertoli Cells

Testicles were isolated from 9-12 day old male BALB/c mouse donors and placed in HBSS, 0.5% BSA (Sigma), on ice. Testicles were chopped in a sterile flow hood with scissors, and digested in a shaking water bath for 6 minutes at 37°C with collagenase (1mg/ml; Sigma Type V) in HBSS. The tissue was washed three times with HBSS and digested in a siliconized 250 ml flask a second time in a shaking water bath for 6 minutes at 37°C with DNase (0.4mg/ml, Boehringer Mannheim, Laval, Canada) and trypsin

(1mg/ml, Boehringer) in calcium-free media containing 1mmol/l EGTA and 0.5% BSA (Sigma). Following the second digest the cells were washed with HBSS and filtered through a 500μm nylon mesh, washed an additional three times and resuspended in 30mls of serum-free HAM's F-10 culture media (no BSA) supplemented with 100 U/ml penicillin/100 U/ml streptomycin (P/S), and cultured in tissue culture-treated plates for 3 days at 37°C and 5% CO_2.

3. Preparation of Sertoli Cell Conditioned Media

Following 3 days of culture at 37°C, the supernatant was collected from cultured Sertoli cells and spun two times for 5 minutes each at 2000 RPM to remove any cellular debris. The resulting Sertoli cell conditioned media (SCCM) was then concentrated with an AmiconYM-10 Centricon devise (10 kDa molecular weight cut-off; Fisher Scientific, Ottawa, Ontario) for 90 minutes at 7000 RPM (4°C). Serum-free HAM's F-10 Sertoli cell culture media (no BSA) was concentrated in a similar manner to SCCM to be used as a control in proliferation assays. Samples were pooled and protein concentration determined with a Bio-Rad dye-binding, protein assay (BioRad Laboratories, Hercules, California) with absorbances measured on an MRX micro plate reader (Dynex Technologies, Chantilly, Virginia) at 600nm. SCCM was stored at 4°C and used fresh in assays.

4. Isolation of Lymphocytes from Lymph Nodes

Lymph nodes were harvested from B6 mice and crushed between two sterile glass slides in a Petri dish with HBSS. Cells were then transferred from the Petri dish to a 50ml tube and allowed to sit on ice for 5 minutes to allow any tissue debris to settle. Supernatant was then transferred to a new 50ml tube and spun down at 1500 RPM for 5

minutes. Cells were then resuspended in 10mls of Earl's minimum essential medium (EMEM) supplemented with 1% L-glutamine, 0.1% 2-mercaptoethanol, 1% P/S, and 10% fetal calf serum and used as responder cells in proliferation assays.

5. Lymphocyte Proliferation Assay

Cells were added to 96-well plates with EMEM media and various treatment conditions in a total volume of 200µl/well. Responder B6 cells were added at a concentration of 2×10^5 cells/well (100µl) either alone (100µl of EMEM), or in combination with the following treatments: 1) SCCM (20µl), and EMEM (80µl), 2) HAM's F10 (20µl), and EMEM, 3) TGF-β (2µl, 50ng/ml, recombinant human latent TGF-β1, R&D Systems, Minneapolis, Minnesota), and EMEM, 4) Concanavalin-A (Con-A, Sigma, 20µl), and EMEM, 5) Con-A, SCCM and EMEM, 6) Con-A, HAM's F10 and EMEM, 7) Con-A, TGF-β, and EMEM. Wells with media alone were also included. Three different 96-well plates with the above mentioned conditions were prepared, with all reagents added at the same time, and incubated for 3, 4, or 5 days at 37°C and 5% CO_2. At the end of each incubation period, plates were pulsed with ^{3}H-thymidine (10µl/well or 1µCi/well, Amersham Biosciences, Piscataway, New Jersey) and incubated for 6 hours at 37°C and 5% CO_2 to allow ^{3}H-thymidine incorporation into proliferating cells. Cells were then harvested (Harvester 96 Mach III M, Tomtec, Hamden, Connecticut) from 96-well plates onto filtermats (Perkin Elmer Life Sciences, Woodbridge, Ontario) and the amount of radioactivity was measured with a beta counter (1450 Microbeta liquid scintillation and luminescence counter, Wallac, Turku, Finland) as counts per minute (CPM) of beta emission which was considered proportional to the amount of cell proliferation.

6. Statistical Analysis

Data is expressed as the median value of triplicate samples. Data in the first graph (Figure 3-1) is representative of Con-A-stimulated B6 mouse lymphocyte proliferation in the presence of HAM's F-10 control media. Data in the second graph (Figure 3-2) is shown as Con-A-stimulated B6 mouse lymphocyte proliferation with HAM's F-10 or EMEM control media subtracted from proliferation in the presence of three different preparations of SCCM or TGF-β. The data shown in the second graph for TGF-β is representative.

III-C) RESULTS

1. The Effect of BALB/c Sertoli Cell Conditioned Media on Con-A-Stimulated B6 Lymphocyte Proliferation

Con-A-stimulated B6 lymphocyte proliferation, in control HAM's F-10 media with EMEM, decreased over time with additional days in culture (Figure 3-1). BALB/c SCCM both decreased and increased Con-A-stimulated B6 lymphocytes proliferation in a manner that was dependent on SCCM preparation and day of lymphocyte culture (Figure 3-2). SCCM appeared to suppress Con-A-stimulated lymphocyte proliferation on day 3 and induce proliferation on subsequent days 4 and 5. However, one preparation of SCCM (prep3) consistently reduced Con-A-stimulated lymphocyte proliferation on all days in culture. SCCM prep2 had a least twice as much protein (128μg/ml) as prep1 (51.2μg/ml) and as such the amplitude of suppression and stimulation of proliferation was greater for this preparation of SCCM. TGF-β consistently decreased Con-A-stimulated B6 lymphocyte proliferation as expected. All SCCM preparations had a slight proliferative effect on B6 lymphocytes in the absence of Con-A.

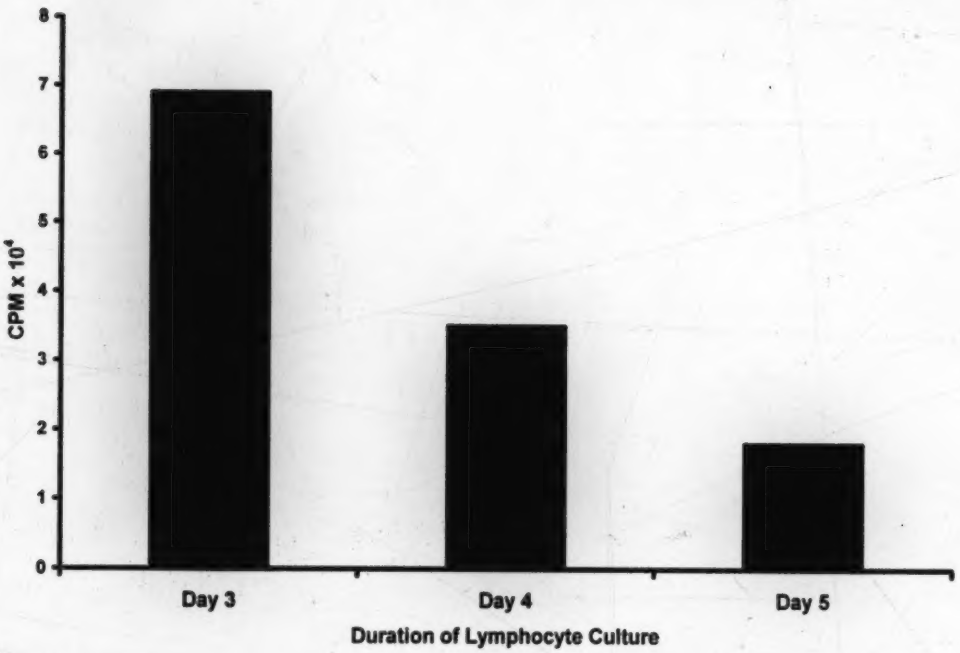

Figure 3-1. Con-A-stimulated proliferation of B6 mouse lymphocytes cultured for 3, 4, or 5 days in the presence of HAM's F-10 control media and EMEM. Lymphocyte proliferation is represented as counts per minute (CPM) of ^{3}H-thymidine uptake by the cells. Background B6 lymphocyte proliferation in the absence of Con-A was subtracted from the values shown. Data is representative.

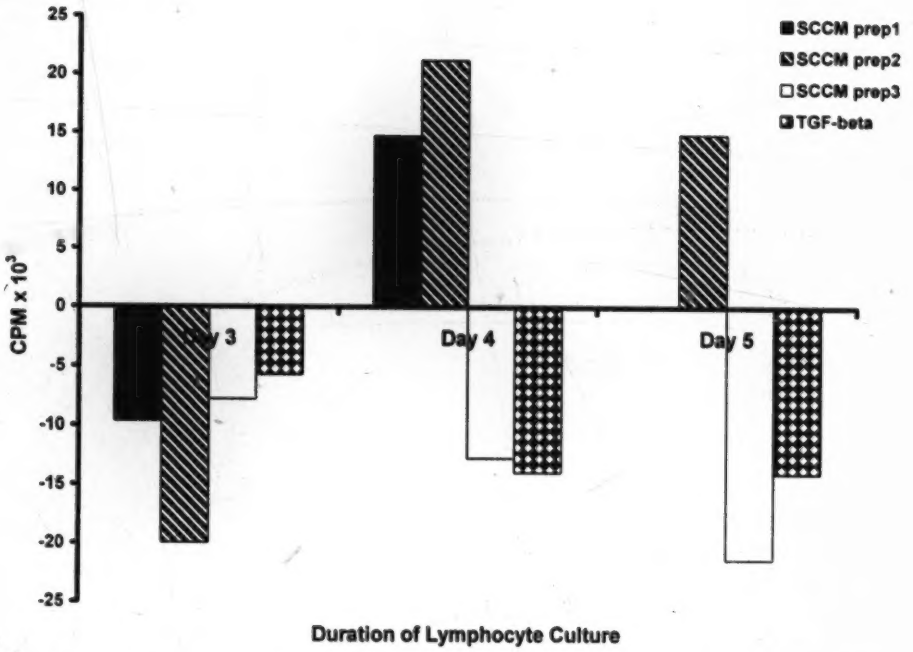

Figure 3-2. Con-A-stimulated proliferation of B6 mouse lymphocytes cultured for 3, 4, or 5 days in the presence of three different preparations (prep 1-3) of BALB/c mouse Sertoli cell-conditioned media (SCCM, 51.2μg/ml, 128μg/ml, and 57.6μg/ml for preps 1-3, respectively) or TGF-β (50ng/ml). Lymphocyte proliferation is represented as counts per minute (CPM) of ^{3}H-thymidine uptake by the cells. Data is represented as Con-A-stimulated lymphocyte proliferation in the presence of HAM's F-10 or EMEM media alone subtracted from Con-A-stimulated lymphocyte proliferation in the presence of SCCM and TGF-β. Furthermore, background B6 lymphocyte proliferation in the absence of Con-A was subtracted from this difference. Negative values on the graph depict a reduction in lymphocyte proliferation by the respective treatment and positive values represent an increase in proliferation due to treatment. Note that proliferation on Day 5 with SCCM prep 1 is not shown because Con-A-stimulated proliferation was too low. TGF-β data is representative.

III-D) DISCUSSION

In the present study we prepared Sertoli cell conditioned media (SCCM) from cultured Sertoli cells isolated from the testis of 9-12 day old BALB/c mice. Subsequently, we tested SCCM for its ability to inhibit Con-A stimulated lymphocyte proliferation. Both SCCM and Con-A were added at the initiation of lymphocyte culture. We found that SCCM was able to inhibit Con-A stimulated lymphocyte proliferation with inhibition being dependent on SCCM preparation and the length of lymphocyte culture (days). For example, SCCM preparations 1 and 2 inhibited Con-A stimulated lymphocyte proliferation on day 3 of lymphocyte culture and stimulated proliferation on subsequent days 4 and 5. However, SCCM preparation 3 and TGF-β consistently inhibited Con-A stimulated lymphocyte proliferation on all days of lymphocyte culture.

Our finding that mouse SCCM inhibits mitogen stimulated lymphocyte proliferation is consistent with previous studies carried out with SCCM prepared from Sertoli cells isolated from rat testis (12-14). However, we also found that SCCM could enhance Con-A stimulated lymphocyte proliferation in later days of culture. This finding is consistent with previous investigations that also showed SCCM could inhibit or enhance mitogen stimulated lymphocyte proliferation depending on the concentration of SCCM (12). For example, at high SCCM concentrations, the inhibitory effect on mitogen stimulated lymphocyte proliferation is favored; however, at low concentrations of the media, the stimulatory effect is favored. It is thought that this happens because the inhibitory activity is diluted away sooner leaving only the stimulatory activity.

To date, the inhibitory and stimulatory factors in SCCM have not been fully characterized, nor have the T-cell subsets that are affected. The factors in our media

where >10 kDa and at least one preparation consistently inhibited mitogen stimulated lymphocyte proliferation in a manner similar to what has been described for TGF-β (15). However, without selectively removing TGF-β (eg. monoclonal antibody treatment) from SCCM we can not conclude it is involved. Other factors, known to be secreted by Sertoli cells, that have similar inhibitory effects on mitogen stimulated lymphocyte proliferation, include: activin, inhibin, and IGF-I (16;17). Future studies should also investigate which T-cell subsets are affected by SCCM. For example, does SCCM inhibit effector T-cell proliferation and stimulate suppressor T-cell proliferation?

Once these SCCM factors have been identified it will be important to study what additional requirements are important for their secretion. For example, hormones may play an important role in the stimulation of Sertoli cell secreted factors. Follicle stimulating hormone (FSH) in particular was found to be important for stimulating Sertoli cell secretion of immunosuppressive factors (13). FSH has been shown, for example, to decrease TGF-β and increase inhibin production by Sertoli cells (23;24). Other potentially important conditions may be temperature at which Sertoli cells are cultured and the stage of the seminiferous tubule from which Sertoli cells are extracted (13;25).

The precise mechanism of how SCCM may be inhibiting mitogen stimulated lymphocyte proliferation is unknown but has been speculated to involve the inhibition of IL-2 production and responsiveness (13).

In conclusion, we have demonstrated that cultured Sertoli cells, isolated from the testis of 9-12 day old BALB/c mice, do secrete a factor(s) that inhibits Con-A stimulated lymphocyte proliferation. Now that we have tested and confirmed the biological activity of our Sertoli cell conditioned media, future work will involve testing the effect of this

media on cytotoxic T lymphocyte (eg. granzyme B) killing, a main immune mechanism of allograft destruction.

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CHAPTER IV

MOUSE SERTOLI CELL CONDITIONED MEDIA INHIBITS GRANZYME B-MEDIATED KILLING

IV-A) INTRODUCTION

Two major obstacles to the successful use of islet transplantation for the treatment of type 1 diabetes include allograft rejection and autoimmune recurrence, both mediated by cytotoxic T lymphocytes (CTLs) (1-5). CTLs provide essential protection against virus infection and intracellular pathogens; however, they can also cause harm in cases of autoimmune disease, graft rejection, and graft-versus-host disease.

Two main mechanisms of CTL killing have been described: receptor- and granule-mediated (2-5). The principle receptor pathway that has evolved is the Fas-Fas ligand (FasL) pathway (6-8). FasL, expressed on activated CTLs and immune privileged tissues, engages the Fas receptor, expressed on adjacent activated CTLs, causing the Fas-bearing cell to undergo apoptotic cell death, the hallmark feature of which is DNA fragmentation (9-11). This mechanism has evolved to protect immune privileged tissues from harmful immune responses and to dispose of lymphocytes at the end of an immune response (12;13). However, under certain conditions FasL can be used by CTLs to mediate other target cell death.

The major mechanism of CTL-mediated killing that has been extensively studied is the granzyme B pathway (5). When a CTL comes into contact with a target cell it delivers a "lethal hit" of cytolytic molecules, that include perforin and granzyme B,

resulting in target cell death by apoptosis (14-16). Briefly, the CTL-granzyme B pathway involves the calcium-dependent release of granzyme B and perforin, stored in cytoplasmic granules of the CTL, in the direction of the target cell. Granzyme B, a mannose-6 phosphorylated (M6P) protein, binds its receptor, the mannose-6 phosphate/insulin-like growth factor-II (M6P/IGF-II) receptor, on the surface of the target cell and along with perforin is endocytosed by the target cell (17). Once inside the target cell granzyme B remains arrested in the endocytic vesicle, unable to mediate apoptosis, until released into the cytoplasm by perforin or some other sort of lytic agent (eg. adenovirus) (18). In the cytoplasm, granzyme B, a serine proteinase, cleaves pro-caspases at aspartic acid residues, activating them and initiating the caspase cascade to DNA fragmentation and apoptotic cell death (19-23).

Co-transplantation of islets with Sertoli cells, isolated from rodent testis, has been found to successfully protect islets from allo- and autoimmune mechanisms of graft destruction (24-27). However, what remains to be determined is the mechanism of how Sertoli cells achieve this feat. One potential way Sertoli cells may prevent graft rejection is through the inhibition of CTL killing. Sertoli cells have been found to express many proteins which may block the CTL-granzyme B pathway to apoptotic cell death. For example, Sertoli cells have been shown to secrete M6P-glycoproteins, and IGF-II, that are ligands for the M6P/IGF-II death receptor for granzyme B (28;29). Some of these M6P-glycoproteins include: prosaposin, procathepsin L, and transforming growth factor-beta (TGF-β) (28;30). TGF-β, in particular, is a known immunosuppressant agent that has been implicated in the mechanism of how Sertoli cells protect islets from autoimmune destruction in the NOD mouse (31;32). Sertoli cells may block the

granzyme B pathway to apoptotic cell death through the secretion of proteins that are ligands for the M6P/IGF-II receptor. These proteins could downregulate or block the receptor, effectively preventing granzyme B uptake and subsequent target cell killing.

Human Sertoli cells have additionally been found to express human proteinase inhibitor-9 (PI-9), a serpin that is a potent inhibitor of granzyme B (33-36). Serpins inhibit proteolysis through the formation of an essentially irreversible complex with their proteinase (37). PI-9 has been found to inhibit granzyme B in vitro and in vivo and PI-9 transfected cells have been shown to be protected against granzyme B mediated apoptosis (35;36). This may explain why Sertoli cells are an immune privileged tissue. Furthermore, Sertoli cells may protect bystander cells through the secretion of PI-9. Even though these links between Sertoli cells and granzyme B killing exist, no studies have ever been carried out on the effect of Sertoli cell secreted factors on granzyme B mediated killing.

Therefore, the focus of the present study was to prepare Sertoli cell conditioned media from cultured Sertoli cells, isolated from the testis of 9-12 day old BALB/c mice, and test its effect on granzyme B-mediated apoptosis. We additionally, characterized specifically how Sertoli cell secreted factors interact with the granzyme B pathway to cell death. To do this we examined the effect of Sertoli cell conditioned media on granzyme B receptor expression, binding and uptake, and enzymatic activity. We found that Sertoli cells secrete a factor that inhibits granzyme B enzymatic activity through the formation of a stable complex that effectively reduces granzyme B mediated apoptosis. This factor exhibited the characteristics of a serpin but was not murine serine proteinase inhibitor-6 (SPI-6), the murine inhibitor of granzyme B.

IV-B) MATERIALS AND METHODS

1. Animals

Male BALB/c (H-2^d) mice (University of Alberta, Edmonton, Alberta, Canada) were used as Sertoli cell (aged 9-12 days) donors. In addition, male C3H (H-2^k) and B6 (H-2^b) mice (Jackson Laboratories, Bar Harbor, Maine), aged 6-8 weeks, were used as splenocyte donors.

2. Cell Lines and Reagents

L-cells (C3H mouse fibroblast cell line), used as target cells in killing assays, were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Burlington, Ontario) supplemented with 10 % FBS, 2mM L-glutamine, and 100μg/ml P/S. C57 cells (B6 mouse CTL cell line), used as effector cells in killing assays, were generated from splenocytes, isolated from spleen of B6 mice, that were stimulated with C3H mouse spleen cells. C57 cells were grown in RPMI 1640 (Gibco) supplemented with 10% FBS, 10^{-4} M 2-mercaptoethanol, 100μg/ml P/S, 20mM Hepes, and 80units/ml of human recombinant IL2 (RHFM). Cells were maintained at a concentration of 5×10^5 cells/ml and were stimulated every Friday with irradiated C3H splenocytes (2500 rads) at a ratio of 1 (C57) to 14 (C3H).

Human purified granzyme B (hp grB), used in TUNEL, and granzyme B enzymatic activity assays, as well as Western blot analysis, was prepared from cytolytic granules of YT INDY cells. Human replication deficient adenovirus (Adv) was used in TUNEL assays in place of perforin to allow entry of hp grB into the cytosol of the target cell. Mouse degranulate granzyme B (degran grB) material, used in granzyme B

enzymatic activity assays, was prepared from C57 cells stimulated with anti-mouse CD3 antibody (BD Biosciences Pharmingen, San Diego, California).

3. Isolation of Mouse Sertoli Cells

Testicles were isolated from 9-12 day old male BALB/c mouse donors and placed in HBSS with 0.5% BSA (Sigma), on ice. Testicles were chopped in a sterile flow hood with scissors, and digested in a shaking water bath for 6 minutes at 37°C with collagenase (1mg/ml; Sigma Type V) in HBSS. The tissue was washed three times with HBSS and digested in a siliconized 250 ml flask a second time in a shaking water bath for 6 minutes at 37°C with DNase (0.4mg/ml, Boehringer Mannheim, Laval, Canada) and trypsin (1mg/ml, Boehringer) in calcium-free media containing 1mmol/l EGTA and 0.5% BSA (Sigma). Following the second digest the cells were washed with HBSS and filtered through a 500μm nylon mesh, washed an additional three times and resuspended in 30mls of serum-free HAM's F10 culture media supplemented with 0.5% BSA (no BSA was added when Sertoli cell-conditioned media was prepared for Western Blot analysis) and 100 U/ml penicillin/100 U/ml streptomycin, and cultured in tissue culture-treated plates for 3 days at 37°C and 5% CO_2.

4. Preparation of Sertoli Cell-Conditioned Media

Following 3 days of culture at 37°C, the supernatant was collected from cultured Sertoli cells and spun two times for 5 minutes each at 2000 RPM to remove any cellular debris. The resulting Sertoli cell-conditioned media (SCCM) was then concentrated with an AmiconYM-10 Centricon devise (molecular weight cut-off of 10 kDa; Fisher Scientific, Ottawa, Ontario) for 90 minutes at 7000 RPM (4°C). Serum-free HAM's F10 Sertoli cell culture media with and without 0.5% BSA, was concentrated in a similar

manner to SCCM to be used as a control in assays and Western Blot analysis. SCCM was stored at 4°C and used fresh in assays.

5. TUNEL Assay for Granzyme B Mediated Apoptosis

A TdT-mediated dUTP nick end labeling (TUNEL) assay was used to measure the amount of DNA fragmentation, a hallmark feature of apoptosis, that occurred in target cells upon incubation with granzyme B. In a TUNEL assay, DNA polymerase and deoxynucleotidyl transferase are used to introduce fluorescein labeled nucleotides into degraded DNA. Subsequently, cells containing labeled DNA are detected by flow cytometry. The percentage of positively labeled target cells, detected using flow cytometry, represents the number of cells undergoing apoptosis.

Fibroblast L-cells were added to a 96-well plate at a concentration of 2×10^5 cells/well. Concentrated SCCM and HAM's F-10 (control media) were both diluted by ½ with fresh DMEM media with 0.1% BSA. SCCM or HAM's F-10 media (25µl/well) were then added to L-cells in the 96-well plate and incubated for 15-30 minutes at 37°C. Following pre-incubation with SCCM or control HAM's F-10 media, plates were spun at 1500 RPM for 5 minutes and cells were given the following treatments: 1) HAM's F-10 or SCCM (25µl), 2) HAM's F-10 or SCCM (22µl) and hp grB (3µl, 600ng/ml), 3) HAM's F-10 or SCCM (20µl) and Adv (5µl, 100 pfu/well), 4) HAM's F-10 or SCCM (17µl) and hp grB (3µl, 600ng/ml) and Adv (5µl, 100 pfu/well), 5) HAM's F-10 or SCCM (14µl) and hp grB (6µl, 120ng/ml) and Adv (5µl, 100 pfu/well), 6) HAM's F-10 or SCCM (18.8µl) and hp grB (1.2µl, 24ng/ml) and Adv (5µl, 100 pfu/well). Once prepared, plates were incubated for 3 hours at 37°C. Following the incubation, L-cells were washed with phosphate buffered saline (PBS) supplemented with 2% FBS and fixed

in PBS with 2% paraformaldehyde and 1% FBS (180µl) overnight at 4°C. Following the overnight fixation procedure, L-cells were washed several times with PBS/2% FBS prior to permeabilization with PBS containing 0.1% saponin (180µl) for 1 hour at room temperature. Cells were then washed again several times with PBS/2% FBS before the addition of TUNEL mix (20µl, Roche Diagnostic, Laval, Quebec) and incubation for 1.5 hours at 37°C. Following incubation with TUNEL mix, cells were washed again in PBS/2% FBS and finally resuspended in 180µl of PBS/2% FBS and transferred from the 96-well plate to 5ml falcon tubes for fluorescence activated cell sorter (FACS, FACS scan, BD Biosciences) analysis of the percentage of TUNEL positive cells.

6. CTL Killing Assay

In a CTL killing assay, the DNA of target cells is labeled with ^{3}H-thymidine. Labeled target cells are incubated with CTL effector cells resulting in target cell DNA fragmentation. The target cells are lysed open, spun down (to pellet cellular debris and intact DNA), and the supernatant containing the fragmented labeled DNA is collected and measured using a beta counter. The percentage of ^{3}H-thymidine released is a measure of DNA fragmentation and indication of the number of cells undergoing apoptosis.

The day before the assay a flask of L-cells (30mls, 4 x 10^{6} cells) was set up and 30µCi of ^{3}H-thymidine (30µl, Perkin Elmer, Billerica, Massachusetts) was added to the flask which was subsequently incubated for 24 hours at 37°C to allow the L-cells to divide and incorporate the ^{3}H-thymidine label into their DNA. The following day the L-cells were removed from the flask with trypsin and transferred to a 50ml tube. L-cells were washed and resuspended in 8mls of DMEM media and allowed to sit for 1 hour at

37°C. At this point L-cells were washed two times with DMEM media, counted, and resuspended in an appropriate volume of DMEM to give 5 x 10^5 cells/ml. L-cells were then transferred to 15ml tubes (1ml/tube) and spun down at 1500 RPM for 3 minutes. L-cells were resuspended in 1ml of DMEM media, HAM's F-10 media, or SCCM and incubated for 1 hour at 37°C. Following pre-incubation with SCCM or control media, L-cells were transferred to eppendorf tubes (100µl/tube) in triplicate to serve as targets for C57 CTL effector cells.

C57 cells were counted, and resuspended in an appropriate volume of RHFM media to give a cell concentration of 5 x 10^6 cells/ml. C57 effector cells were then mixed with L-cells in eppendorf tubes (100µl/tube) at a ratio of 10:1 (effector to target cell ratio). Some tubes contained L-cells with RHFM media alone and no effector C57 cells to determine the amount of spontaneous ^{3}H-thymidine released from cells. Once mixed, cells were incubated for 3 hours at 37°C. During this incubation period the total amount of ^{3}H-thymidine in labeled L-cells was determined.

Briefly, L-cells (50µl) were added to liquid scintillation vials in triplicate along with totals lysis buffer (2% sodium dodecyl sulfate, SDS, 400µl). Samples were mixed for 1 minute using a vortex machine and allowed to sit for 2 hours at room temperature (to lyse open the cells). Totals samples were then mixed again with a vortex machine and 5mls of aqueous counting scintillant (Amersham Biosciences, Baie d'Urfe, Quebec) was added to each vial which was subsequently read using a beta counter (Beckman LS7800, Mississauga, Ontario).

Following the 3 hour incubation, samples of target and effector cells were prepared for determination of ^{3}H-thymidine release. Sample lysis buffer (1% Triton X,

200µl) was added to each eppendorf tube containing the samples and tubes were mixed for 1 minute using a vortex machine. Tubes were subsequently spun at 1400 RPM for 10 minutes at 4°C. Supernatants (200µl) were transferred to liquid scintillation vials and aqueous counting scintillant (5mls) was added. Samples were then placed in a beta counter for the determination of amount of ^{3}H-thymidine release. The percent specific ^{3}H-thymidine release per sample was calculated as follows: [(sample count [target and effector] - spontaneous count [target alone]) / (totals count – spontaneous count)] x 100.

7. FACS Staining for CI- and CD-MPR

Two forms of the mannose-6 phosphate receptor (MPR) have been described, the ~270 kDa cation-independent MPR (CI-MPR), also known as the M6P/IGF-II receptor, and the 46 kDa cation-dependent MPR (CD-MPR) (38). The CI-MPR is the main form of the MPR responsible for the binding and internalization of granzyme B but the CD-MPR also binds but does not effectively internalized granzyme B (17). We characterized the effect of SCCM on both forms of the receptor.

L-cells were added to 96-well plates at a concentration of 2 x 10^{5} cells/well and spun at 1500 RPM for 5 minutes. Subsequently, L-cells were pre-incubated with SCCM or HAM's F-10 control media for 1 hour at 37°C. For CI-MPR and CD-MPR staining, L-cells were incubated for 1 hour at 4°C with either 1) PBS with 0.1% BSA (50µl, control), or 2) rabbit anti-bovine CI-MPR (50µl, 1/500, William Brown, Cornell University), or 3) rabbit anti-human CD-MPR (50µl, 1/100, William Sly, Saint Louis University). Following this incubation, cells were washed with 180µl of PBS with 0.1% BSA and spun at 1500 RPM for 3 minutes. Cells were then incubated for 20 minutes at 4°C with goat anti-rabbit conjugated to Fluorescein Isothiocyanate (FITC, 1/100, Jackson,

Mississauga, Ontario). Following this incubation, cells were washed with PBS supplemented with 2% FBS and fixed in PBS with 2% paraformaldehyde and 1% FBS (180µl) overnight at 4°C. Following the overnight fixation procedure, cells were washed several times with PBS/2% FBS prior to being resuspended in 180µl of PBS/2% FBS and transferred from the 96-well plate to 5ml falcon tubes for FACS (FACS scan, BD Biosciences) analysis of the relative mean fluorescence intensity (MFI). The MFI represents the level of CI- and CD-MPR cell surface expression.

8. Binding and Uptake of Granzyme B

For the detection of granzyme B binding and uptake, target L-cells were incubated with fluorescein labeled granzyme B either at 4°C or 37°C for binding and uptake, respectively. At 4°C the CI-MPR remains arrested at the cell surface (also used for MPR staining) for the detection of granzyme B binding, whereas, at 37°C the CI-MPR gets internalized by the cell and granzyme B uptake can be measure. MFI of labeled cells is measured using flow cytometry with the MFI being proportional to the amount of granzyme B that is bound or taken up by the cell.

L-cells were added to 96-well plates at a concentration of 2×10^5 cells/well and spun at 1500 RPM for 5 minutes. Subsequently, L-cells were pre-incubated with SCCM or HAM's F-10 control media for 1 hour at 37°C. For granzyme B binding to L-cells, cells were incubated for 1 hour at 4°C with either 1) PBS with 0.1% BSA (7µl, control), or 2) PBS with 0.1% BSA (2µl) and granzyme B conjugated to Alexa 488 (5µl, Molecular Probes). For granzyme B uptake into L-cells, cells were incubated for 1 hour at 37°C with either 1) DMEM with 0.1% BSA (25µl, control), or 2) DMEM with 0.1% BSA (20µl) and granzyme B conjugated to Alexa 488 (5µl, Molecular Probes).

Following these incubations, cells were washes with either PBS or DMEM with 0.1% BSA for 4°C and 37°C plates, respectively. Both plates were spun at 1500 RPM for 3 minutes and cells were fixed as described before and transferred to tubes for FACS analysis of MFI.

9. Granzyme B Enzymatic Activity Assay

Granzyme B is a serine proteinase that mediates apoptosis by cleaving substrates (eg. caspases) at aspartic acid residues (19). An assay has been developed in our lab that measures the enzymatic activity of granzyme B. This assay involves the incubation of granzyme B with a tetra peptide solution (isoleucine/glutamate/proline/aspartate [IEPD]) conjugated to paranitroanalide (pNA). IEPD-pNA contains the cleavage site for granzyme B. When IEPD-pNA is cleaved by granzyme B it produces IEPD and pNA, a colored product, whose absorbance can be measured at 405 nm and assumed to be proportional to the amount of granzyme B enzymatic activity in the assay.

Hp grB was diluted in eppendorf tubes with PBS/2% FBS, HAM's F-10 media, or SCCM to give concentrations of 600ng/ml, 120ng/ml, and 24ng/ml of hp grB. Samples were then transferred to a 96-well plate in triplicate (50µl/well). Some wells received PBS/2% FBS, HAM's F-10, or SCCM media without hp grB to serve as background controls (50µl/well). In a second 96-well plate, mouse degran grB (25µl) was added in combination with PBS/2% FBS, HAM's F-10 media, or SCCM (25µl) in triplicate. Some wells contained PBS/2% FBS, HAM's F-10 media, or SCCM (50µl) only and served as background controls. The approximate concentration of the mouse degran grB was determined by preparing a series of standards of hp grB of known concentration. Both plates were then pre-incubated for 30 minutes at 37°C prior to the addition of a tetra

peptide solution, IEPD-pNA (50μl), to wells in both plates. Both plates were incubated for 5 hours at 37°C with readings (Absorbance at 405nm, for the detection of the pNA cleavage product) taken at time zero and every hour thereafter.

10. Western Blot for Granzyme B and SPI-6

A granzyme B stock solution was prepared by adding 5μl of hp grB (120μg/ml) to 45μl of PBS for a final hp grB concentration of 12μg/ml. Samples were prepared in eppendorf tubes as follows: 1) HAM's F-10 (40μl, no BSA), 2) SCCM (40μl, no BSA), 3) HAM's F-10 (37μl) and hp grB (3μl, 12μg/ml), 4) SCCM (37μl) and hp grB (3μl), and 5) PBS (37μl) and hp grB (3μl). The sixth sample was a C57 mouse CTL lysate used as a positive control for SPI-6. These samples were subsequently incubated for 2 hours at 37°C. Following this incubation, sample buffer with 5% sodium dodecyl sulfate (SDS), 0.6% bromophenol blue, and 0.1% 2-mercaptoethanol was added to samples (10μl) which were then denatured by heating at 100°C for 5 minutes. Samples were then run on a 10% SDS-polyacrylamide gel at 30mA/gel for 1.5 hours. Following separation on a 10% SDS-polyacrylamide gel, samples were transferred using electrophoresis to a PVDF membrane (Millipore, Bedford, Massachusetts) which was blotted for granzyme B or SPI-6, stripped and reprobed for granzyme B. PVDF membranes were stripped with 62.5mM Tris-HCl (pH 6.7) containing 2% SDS and 100mM 2-mercaptoethanol for 30 minutes at 60°C in a shaking water bath.

For granzyme B staining, the PVDF membrane was blocked with Tris buffered saline with 0.1% Tween-20 (TBS-T) containing 5% skim milk for 1 hour at room temperature on a rocking platform. The membrane was then washed once with TBS-T containing 1% skim milk prior to the addition of 2C5 mouse monoclonal anti-human

granzyme B antibody (7.5ml, 1:500, Santa Cruz, Santa Cruz, California) diluted with TBS-T containing 5% skim milk. The membrane was incubated with the 2C5 antibody overnight at 4°C on a rocking platform. Following incubation with 2C5 antibody, the membrane was washed four times with TBS-T containing 1% skim milk before the addition of anti-mouse horse radish peroxidase antibody (10ml, 1:3000, Bio Rad, Mississauga, Ontario) diluted with TBS-T containing 5% skim milk. The membrane was incubated with this antibody for 1 hour at room temperature on a rocking platform. The membrane was then washed four times with TBS-T before the addition of ECL (Amersham Biosciences, Piscataway, New Jersey) and film development.

For SPI-6 staining two different antibodies were used, one that was specific for SPI-6 and another that was specific for PI-9 (P19-17) shown to cross-react with SPI-6 (33;39). PVDF membranes were blocked with Tris buffered saline with 0.1% Tween-20 (TBS-T) containing 5% skim milk for 1 hour at room temperature on a rocking platform. The membranes were then washed once with TBS-T containing 1% skim milk prior to the addition of rabbit anti-mouse SPI-6 antibody (7.5ml, 1:5000, J.P. Medema, Leiden University Medical Center, Leiden, The Netherlands) or P19-17 mouse anti-human PI-9 antibody (7.5ml, 8.5µg/ml, Alexis Biochemicals, San Diego, California) diluted with TBS-T containing 5% skim milk. The membranes were incubated with primary antibody overnight at 4°C on a rocking platform. Following incubation with primary antibody, the membranes were washed four times with TBS-T containing 1% skim milk before the addition of anti-rabbit horse radish peroxidase antibody (10ml, 1:20 000, Bio Rad) or anti-mouse horse radish peroxidase antibody (10ml, 1:3000, Bio Rad) diluted with TBS-T containing 5% skim milk. The membranes were incubated with secondary antibody for

1 hour at room temperature on a rocking platform. The membranes were then washed four times with TBS-T before the addition of ECL (Amersham Biosciences, Piscataway, New Jersey) and film development.

11. Statistical Analysis

Data is expressed as the mean ± SEM of at least three independent experiments (done in triplicate samples) with at least three different preparations of Sertoli cell conditioned media. Statistical significance of differences between two independent groups was calculated with a paired t-test. A value of $p < 0.05$ was considered significant.

IV-C) RESULTS

1. The Effect of Sertoli Cell Conditioned Media on Granzyme B-Mediated Killing

DNA fragmentation is a hallmark feature of apoptosis. The TUNEL assay measures the number of cells undergoing DNA fragmentation (% specific TUNEL positive cells) due to apoptotic cell death. Granzyme B treatment of L-cells resulted in dose dependent DNA fragmentation and cell death (Figure 4-1). SCCM effectively reduced granzyme B mediated DNA fragmentation in target L-cells. The reduction in DNA fragmentation was found to be significant at doses of 120 and 600 ng/ml of granzyme B ($p<0.05$).

In the CTL killing assay, ^{3}H-thymidine labeled target cells were incubated with CTL effector cells. Labeled L-cells were then lysed open and spun down to pellet any cellular debris and intact DNA. The amount of fragmented DNA in the cell supernatant was assessed using a beta counter and the percent ^{3}H-thymidine released is considered to

be proportional to the number of cells undergoing apoptosis. CTLs kill target cells through calcium dependent granzyme B killing and calcium independent FasL killing (2;6-8). Ethylene glycol tetra acetic acid (EGTA) binds calcium and eliminates granzyme B killing. We found that EGTA treatment of L-cells incubated with C57 CTL effector cells eliminated all killing suggesting that the main pathway used by C57 cells for target cell killing was the granzyme B pathway. C57 CTL effector cells effectively killed target L-cells (40% specific ^{3}H-thymidine release) at a ratio of 10 effector cells to 1 target cell (Figure 4-2). SCCM significantly reduced C57 CTL killing of L-cells.

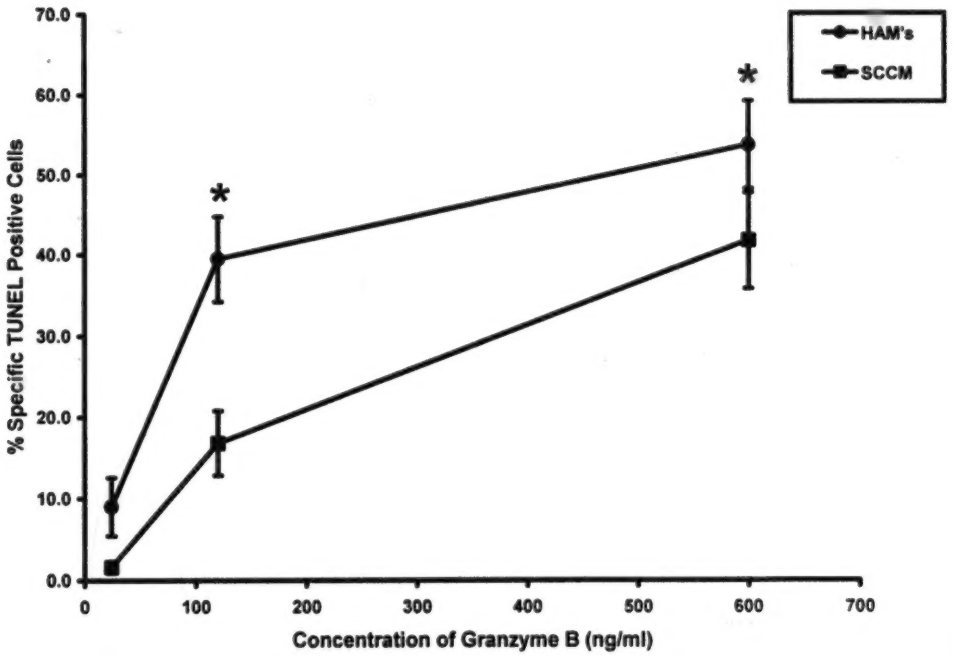


Figure 4-1. The effect of BALB/c mouse Sertoli cell conditioned media (SCCM) on human purified granzyme B-mediated killing of mouse C3H fibroblast L-cells. L-cells were incubated with 24, 120, or 600ng/ml of granzyme B and 100 pfu/cell of replication deficient adenovirus for 3 hours at 37°C in the presence of either HAM's F-10 control media or SCCM. Killing or DNA fragmentation in target cells was assessed using a TUNEL assay. The percent specific TUNEL positive cells (cells that underwent DNA fragmentation), was determined by flow cytometric analysis. Data shown as the mean ± SEM of four independent experiments carried out on four different preparations of SCCM. *Significant reduction ($p<0.05$) in granzyme B-mediated killing upon treatment with SCCM as compared to HAM's F-10 control media.

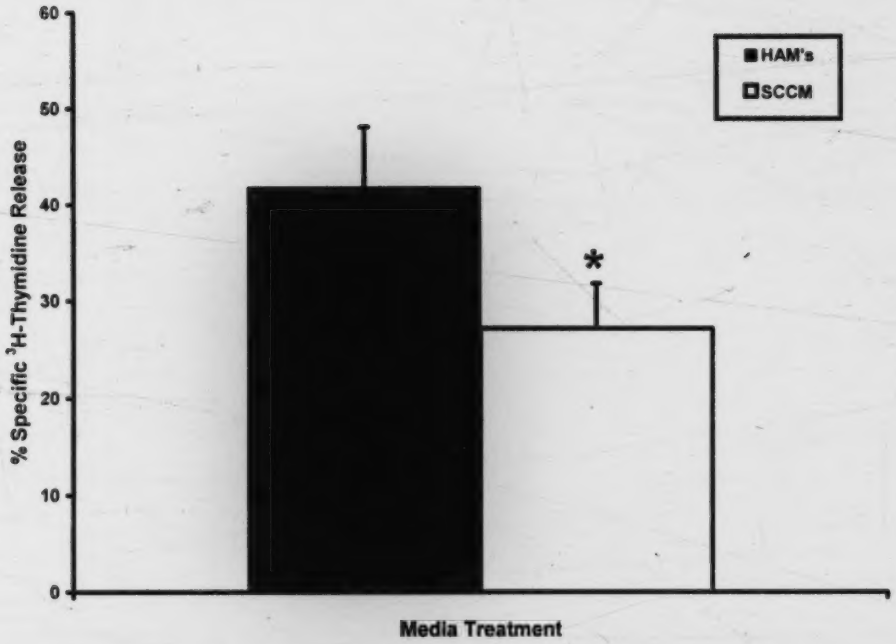
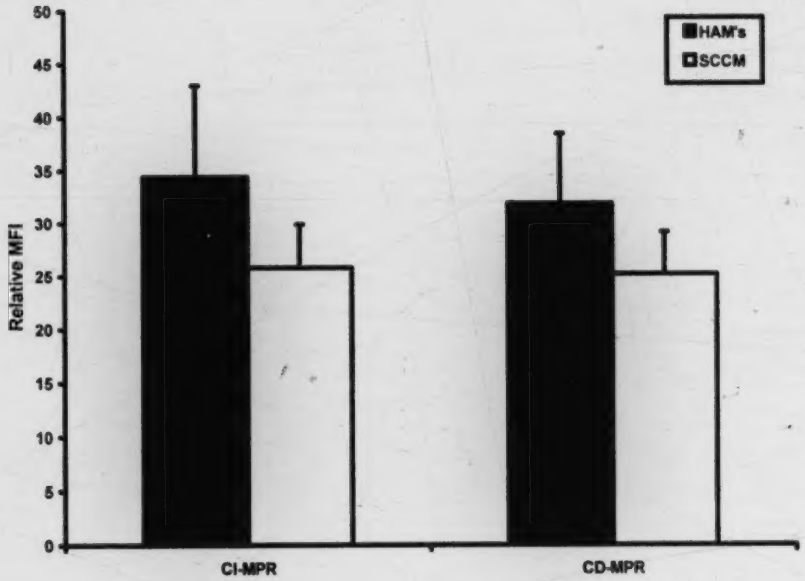


Figure 4-2. The effect of BALB/c mouse Sertoli cell conditioned media (SCCM) on CTL killing of mouse C3H fibroblast L-cells. ^{3}H-thymidine labeled L-cell targets were mixed with effector C57 mouse CTLs at a ratio of 10:1 (effector:target) and incubated for 3 hours at 37°C in the presence of either HAM's F-10 control media or SCCM. Killing or DNA fragmentation was assessed by lysing the target L-cells open, spinning the cells down and collecting the supernatant for assessment of percent specific ^{3}H-thymidine release using a beta counter. Data shown as the mean +SEM of three independent experiments carried out on three different preparations of SCCM. *Significant reduction ($p<0.05$) in CTL killing upon treatment with SCCM as compared to HAM's F-10 control media.

2. The Effect of Sertoli Cell Conditioned Media on Granzyme B Receptor Expression and Uptake by Target Cells

Next, we wanted to determine how SCCM reduces granzyme B mediated apoptosis by testing its effect on granzyme B receptor expression (M6P/IGF-II or CI-MPR) and granzyme B binding and uptake in L-cells. We found that SCCM reduced L-cell surface expression of both forms of the MPR (CI and CD-MPR), however, not significantly (Figure 4-3A). We also found that SCCM had no effect on granzyme B binding and although it did reduce granzyme B uptake by L-cells, this was not found to be statistically significant (Figure 4-3B).

A



B

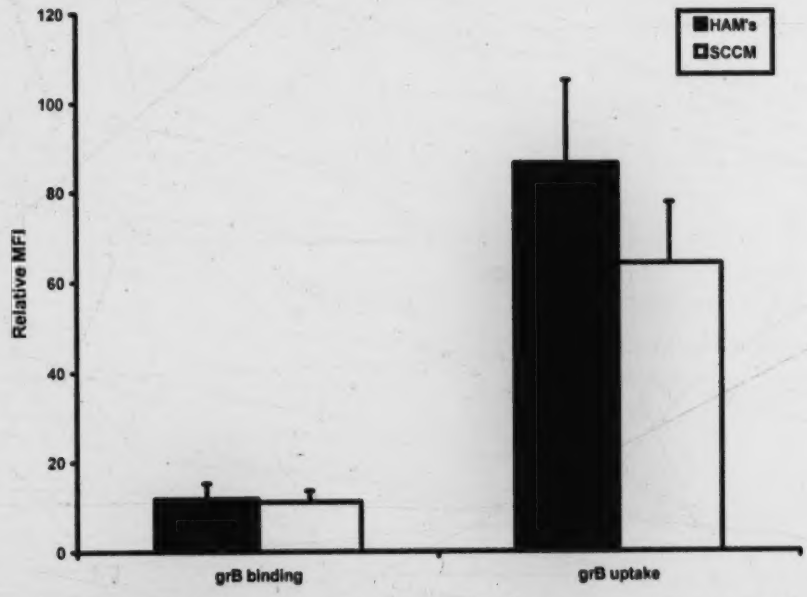


Figure 4-3. Effect of Sertoli cell-conditioned media (SCCM) on (A) the Cation-independent and -dependent (CI/CD) forms of the mannose-6 phosphate receptor (MPR) for granzyme B in L-cells and (B) granzyme B (grB) binding and uptake in L-cells. Data shown as the mean +SEM of three independent experiments carried out on three different preparations of SCCM. MFI-mean fluorescence intensity.

3. The Effect of Sertoli Cell Conditioned Media on Granzyme B Enzymatic Activity

Since no significant effect of SCCM on CI-MPR expression or granzyme B uptake was found we decided to examine the effect of SCCM on granzyme B enzymatic activity. For the determination of granzyme B enzymatic activity, granzyme B was incubated with a tetra peptide solution (IEPD) conjugated to pNA following an aspartic acid residue. Granzyme B cleavage after the aspartic acid residue released pNA, a colored product, whose absorbance was measured at 405 nm. We found that SCCM significantly reduced both human and mouse granzyme B enzymatic activity (Figures 4-4 and 4-5).

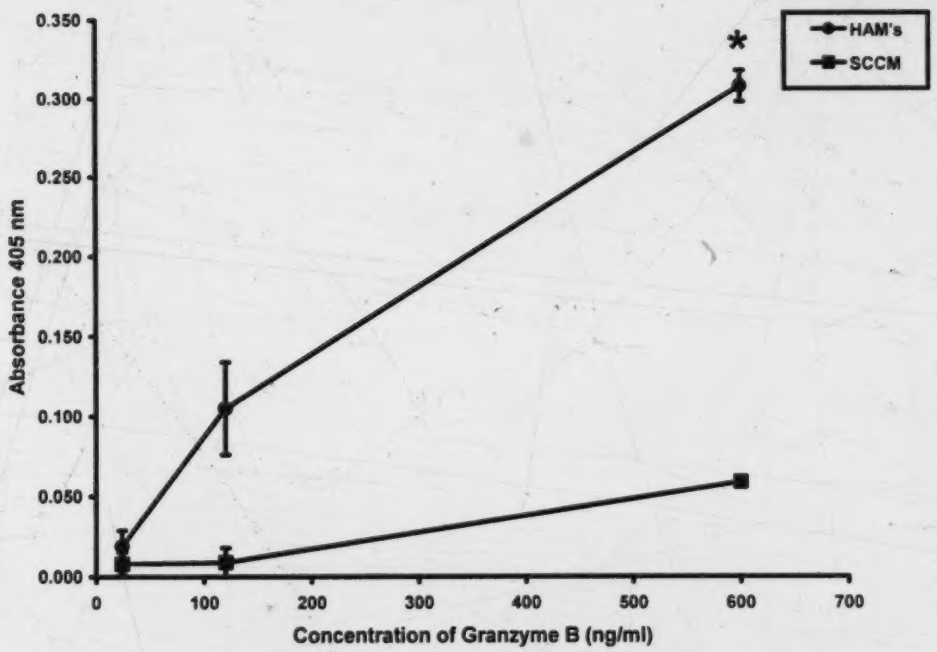


Figure 4-4. The effect of BALB/c mouse Sertoli cell conditioned media (SCCM) on human purified granzyme B enzymatic activity. Granzyme B enzymatic activity was assessed for 24, 120, and 600ng/ml of granzyme B in the presence of either HAM's F-10 control media or SCCM and IEPD-pNA. When cleaved IEPD-pNA produces IEPD + pNA, a colored product, whose absorbance was measured at 405 nm and assumed to be proportional to the amount of granzyme B enzymatic activity in the assay. Data shown as the mean ± SEM of three independent experiments carried out on three different preparations of SCCM. *Significant reduction ($p<0.05$) in granzyme B enzymatic activity upon treatment with SCCM as compared to HAM's F-10 control media.

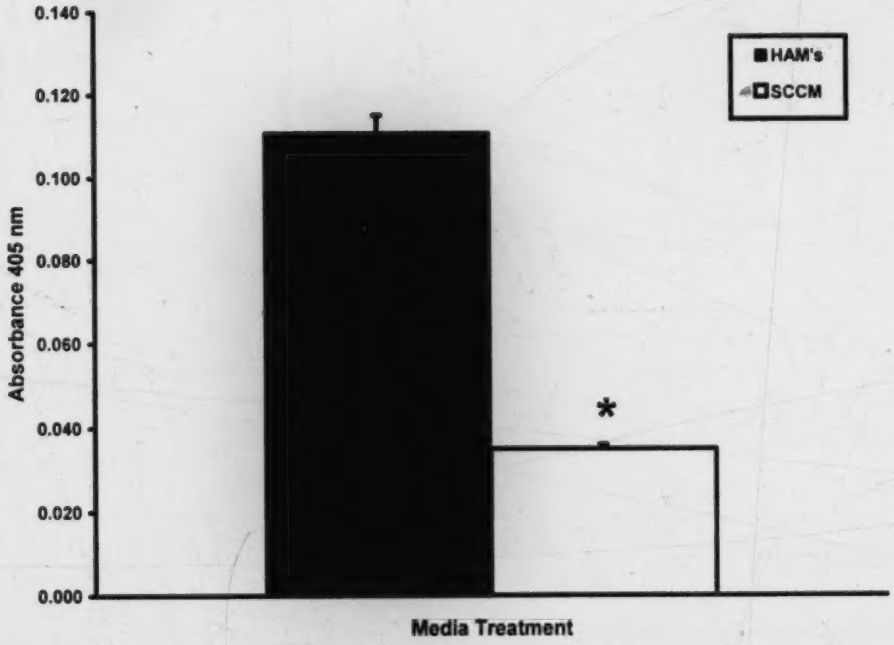


Figure 4-5. The effect of BALB/c mouse Sertoli cell-conditioned media (SCCM) on C57 CTL mouse granzyme B degranulate enzymatic activity. Granzyme B enzymatic activity was assessed for approximately 2.88ng/ml of degranulate granzyme B in the presence of either HAM's F-10 control media or SCCM and IEPD-pNA. When cleaved IEPD-pNA produces IEPD + pNA, a colored product, whose absorbance was measured at 405 nm and assumed to be proportional to the amount of granzyme B enzymatic activity in the assay. Data shown as the mean +SEM of three independent experiments carried out on three different preparations of SCCM. *Significant reduction ($p<0.05$) in granzyme B enzymatic activity upon treatment with SCCM as compared to HAM's F-10 control media.

4. Identification of the Factor in Sertoli Cell Conditioned Media that Affects Granzyme B Enzymatic Activity and Killing

For the identification of the factor in SCCM that inhibits granzyme B enzymatic and biological activity, we co-cultured SCCM with human purified granzyme B and ran the sample on a 10% SDS-polyacrylamide gel. The samples were transferred to a PVDF membrane and stained with 2C5 anti-human granzyme B antibody for the detection of granzyme B. Lanes with HAM's F-10 control media alone (lane 1) and SCCM alone (lane 2) showed no banding patterns (Figure 4-6), as expected, as no granzyme B was added to those samples. Lanes with HAM's F-10 and granzyme B (lane 3) and granzyme B alone (lane 5) showed a band at approximately 32 kDa, characteristic of granzyme B, and a second band between 50 and 64 kDa that was most likely a glycosylated form of granzyme B. The lane with SCCM and granzyme B (lane 4) showed the typical 32 kDa band, characteristic of granzyme B, but the higher molecular weight form of granzyme B between 50 and 64 kDa was gone and instead a band between 64 and 98 kDa appeared.

We suspected that the SCCM factor responsible for the inhibition of granzyme B enzymatic and biological activity was a serine proteinase inhibitor or serpin. Serpins are known to bind essentially irreversibly to their proteinase in a manner that is resistant to boiling and SDS, a property thought to be unique among this class of proteinase inhibitors (37). Similarly, we observed the formation of a stable complex between the SCCM factor and granzyme B in our Western blots. The serine proteinase inhibitor known to inhibit granzyme B enzymatic activity through the formation of a stable complex is murine SPI-6. Sertoli cells have additionally been found to express the human version of SPI-6, PI-9, a potent inhibitor of human granzyme B (33;40). For these reasons we decided to do Western blots, probing for SPI-6 in the observed complex.

Samples were prepared as before with the addition of mouse CTL lysate as a positive control for SPI-6. Staining with anti-mouse SPI-6 antibody essentially showed no bands except for in lane 6 with the mouse CTL lysate. There the characteristic 42 kDa band for SPI-6 was observed along with other higher molecular weight bands between 36 and 50 kDa and between 50 and 64 kDa (Figure 4-7A). When this blot was stripped and probed for granzyme B, the complex between 64 and 98 kDa was observed in lane 4 with SCCM and granzyme B (Figure 4-7B). Likewise, when a second antibody was used for the detection of SPI-6 (anti-human PI-9 antibody that cross-reacts) bands appeared in lanes with SCCM and in the lane with mouse CTL lysate. These bands all came out at the same level with the typical 42 kDa band for SPI-6 and other higher molecular weight bands between 50 and 64 kDa (Figure 4-7C). When this blot was stripped and probed for granzyme B, the complex between 64 and 98 kDa was observed in lane 4 containing SCCM and granzyme B (Figure 4-7D). These findings indicate that SPI-6 is not the serpin in the observed complex of SCCM and granzyme B as no higher molecular weight bands in the SPI-6 blot corresponded with granzyme B in the granzyme B blot.

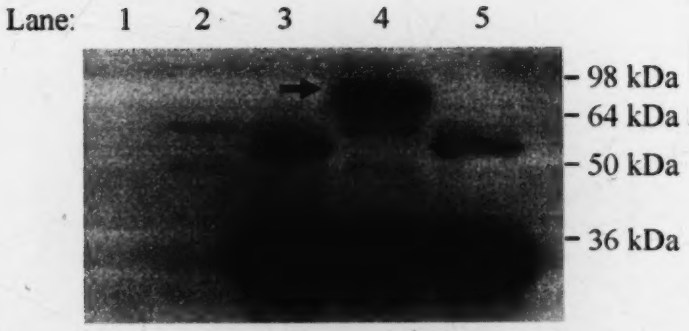


Figure 4-6. Identification of a factor in BALB/c mouse Sertoli cell-conditioned media (SCCM) that complexes with human purified granzyme B. Human purified granzyme B was incubated with HAM's F-10 control media, SCCM, or PBS for 2 hours at 37°C. Following the incubation, 5x SDS sample buffer (10µl) was added to samples and samples were boiled for 5 minutes at 100°C prior to electrophoresis on a 10% SDS-polyacrylamide gel, transfer to PVDF membrane, and staining with 2C5 mouse monoclonal anti-human granzyme antibody for the detection of granzyme B. Lanes represent the following samples: 1- HAM's, 2- SCCM, 3- HAM's with granzyme B, 4- SCCM with granzyme B, and 5- granzyme B. The arrow (lane 4) indicates an unidentified higher molecular weight band that forms when SCCM and granzyme B are combined. The molecular weight of granzyme B is 32 kDa.

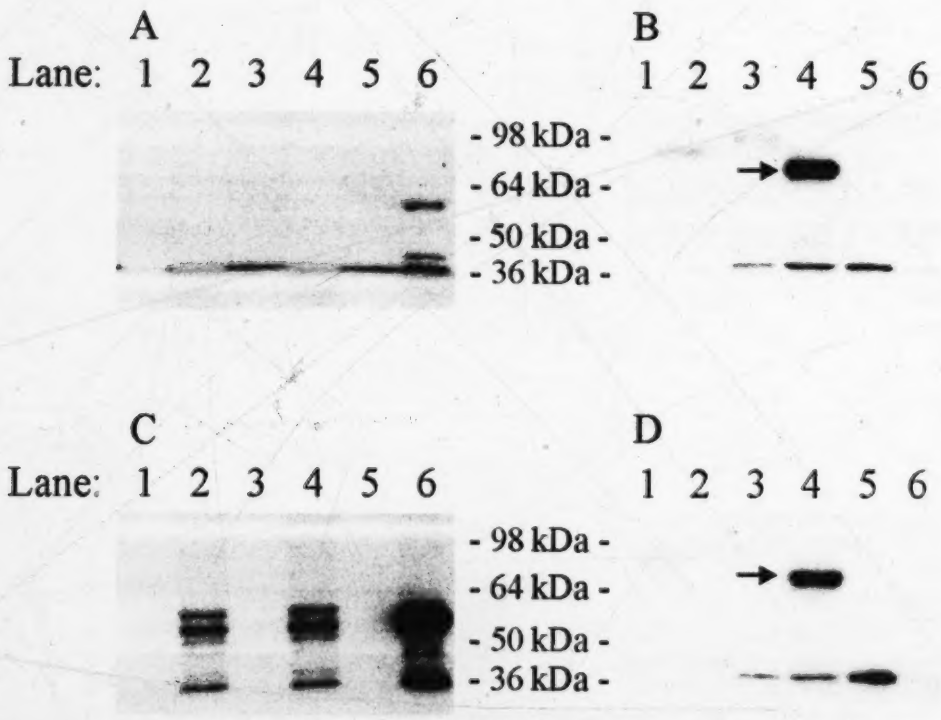


Figure 4-7. Identification of a factor in BALB/c mouse Sertoli cell-conditioned media (SCCM) that complexes with human purified granzyme B. Human purified granzyme B was incubated with HAM's F-10 control media, SCCM, or PBS for 2 hours at 37°C. Following the incubation, 5x SDS sample buffer (10µl) was added to samples and samples were boiled for 5 minutes at 100°C prior to electrophoresis on a 10% SDS-polyacrylamide gel, transfer to PVDF membrane, and staining with (A) rabbit anti-mouse SPI-6 antibody for the detection of SPI-6, (B) blot in (A) stripped and reprobed with 2C5 mouse monoclonal anti-human granzyme B antibody for the detection of granzyme B, (C) P19-17 mouse anti-human PI-9 antibody for the detection of SPI-6, (D) blot in (C) stripped and reprobed with 2C5 mouse monoclonal anti-human granzyme B antibody for the detection of granzyme B. Lanes represent the following samples: 1- HAM's, 2- SCCM, 3- HAM's with granzyme B, 4- SCCM with granzyme B, 5- granzyme B, and 6- mouse CTL lysate. The arrow (lane 4) indicates a higher molecular weight band that forms when SCCM and granzyme B are combined. The SCCM factor that is stably bound to granzyme B does not appear to be SPI-6 but may be another yet unidentified serpin. The molecular weight of granzyme B and SPI-6 are 32 kDa and 42 kDa, respectively.

IV-D) DISCUSSION

Sertoli cells are known to protect islets from auto- (27), allo- (24-26), and even xenoimmune (41;42) mechanisms of graft destruction and it has been suggested, although not proven in every situation, that Sertoli cells protect bystander cells from immune destruction through the secretion of immunosuppressive factors (32). For example, Sertoli cell mediated protection of islets in the NOD mouse model, a model of autoimmune diabetes, has been attributed to TGF-β secreted by Sertoli cells (32). TGF-β is an anti-inflammatory cytokine capable of suppressing T-cell, macrophage, natural killer cell, and B cell activity as well as the expression of many proinflammatory cytokines (31). In the present study we show that Sertoli cells secrete factors that inhibit CTL/granzyme B mediated apoptosis, a main immune effector mechanism in graft destruction.

Subsequently, we wanted to understand how Sertoli cells inhibit granzyme B mediated target cell killing. Previous studies had demonstrated that Sertoli cells communicate with developing spermatogonia through the secretion of numerous M6P-glycoproteins that interact with cell-surface M6P/IGF-II receptors (29). The cell-surface M6P/IGF-II receptor has also been shown to bind and internalize granzyme B during CTL mediated target cell killing (17). Once inside the target cell, granzyme has been shown to mediate rapid DNA fragmentation, a hallmark feature of apoptosis (21). We suspected that Sertoli cells may be inhibiting granzyme B mediated apoptosis through the secretion of ligands for the M6P/IGF-II receptor. However, surprisingly, we found that factors secreted by Sertoli cells had no effect on M6P/IGF-II receptor cell-surface expression, nor did these factors interfere with granzyme B binding or uptake. As a

result, we suspected that maybe a factor secreted by Sertoli cells was inhibiting granzyme B enzymatic activity. Indeed, we did find that a factor secreted by mouse Sertoli cells effectively reduced both human and mouse granzyme B enzymatic activity. However, the question that remained was what factor secreted by Sertoli cells might be affecting granzyme B enzymatic activity.

Human proteinase inhibitor-9 (PI-9) is a potent inhibitor of granzyme B enzymatic activity (35;36). PI-9 was originally found to be expressed by T-cells and proposed to protect CTLs against death mediated by misdirected granzyme B (35). PI-9 may play a similar protective role in Sertoli cells, recently found to also express granzyme B (33;34). PI-9 inhibits granzyme B enzymatic activity through the formation of an SDS stable complex with granzyme B (37). We incubated human granzyme B with mouse SCCM, ran the samples on an SDS-polyacrylamide gel electrophoresis, and performed a Western blot for granzyme B. In the lane with SCCM and granzyme B we saw the appearance of a unique band in the molecular weight range of 64-98 kDa. Because the SCCM factor bound to granzyme B in a manner that was resistant to SDS we suspected that it might be a serine proteinase inhibitor (serpin). Additionally, mouse serine proteinase inhibitor-6 (SPI-6) has been found to form a complex with granzyme B in the same molecular weight range as the unidentified complex (67 kDa) (43). Since our SCCM was prepared from Sertoli cells isolated from the testis of mouse we decided to check if the unidentified complex consisted of SPI-6 bound to granzyme B. We used two different antibodies for the detection of SPI-6 in the complex and did not see any corresponding band in the SPI-6 blots that were stripped and subsequently blotted for granzyme B. This led us to believe that some other serpin secreted by Sertoli cells must

be interacting with granzyme B to form an SDS stable complex. To date we have been unable to identify this factor.

In conclusion, we found that a factor secreted by Sertoli cells inhibits granzyme B enzymatic and biological killing activity. This factor remains elusive but is hypothesized to be a serpin due to the stable nature of interaction of this factor with granzyme B under reducing conditions. Future work should involve the identification of this factor using mass spectroscopic analysis. In any event these experiments have brought us closer to unlocking the secrets of how Sertoli cells protect islet allografts from immune destruction; knowledge that might be used to further optimize co-transplantation conditions in the future and pave the way for the use of Sertoli cells in clinical islet transplantation.

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CHAPTER V

CHARACTERIZATION OF THE MANNOSE-6 PHOSPHATE/INSULIN-LIKE GROWTH FACTOR-II RECEPTOR FOR GRANZYME B IN DISSOCIATED MOUSE ISLETS AND PANCREACTIC CELL LINES

V-A) INTRODUCTION

A major barrier to successful islet transplantation is allograft rejection (1). The main immune mediator in graft rejection is the cytotoxic T lymphocyte (CTL) that destroys its target cell through the release of cytolytic molecules such as perforin and granzymes (2-5). It has been discovered recently that the mannose-6 phosphate/insulin-like growth factor-II (M6P/IGF-II) receptor is a death receptor that binds and internalizes granzyme B during CTL killing (6). Once inside the target cell granzyme B is able to mediate DNA fragmentation and apoptotic cell death (7). This discovery potentially has profound repercussions in that any therapeutic developed to block the interaction of granzyme B with its receptor may prevent allograft rejection.

There are two main types of the mannose 6-phosphate receptor (MPR), the 270 kDa cation-independent MPR (CI-MPR) and the 46 kDa cation-dependent MPR (CD-MPR) (8). A main role for the MPRs has been described in lysosomal enzyme trafficking within the cell; however, MPRs have also been found to come to the cell surface to bind and internalize mannose 6-phosphorylated (M6P)-proteins (9;10). The CI-MPR, also known as the M6P/IGF-II receptor, is the main form of the receptor responsible for the binding and uptake of granzyme B (6). The CD-MPR can bind but does not effectively

internalize granzyme B. Previous studies have examined MPR cell surface expression and granzyme B binding as well as uptake in the fibroblast L-cell line; however, no studies have examined the granzyme B-MPR pathway to cell death in primary islets or pancreatic cell lines.

Therefore, the goal of the present study was to characterize CI- and CD-MPR expression as well as granzyme B binding and uptake in dissociated mouse islets and two different pancreatic cell lines, rat INS-1 β-cell line and hamster InR1G9 α-cell line. Once the importance of the granzyme B-MPR pathway has been established in islets, therapeutics can be developed to block this pathway and potentially prevent islet allograft rejection.

V-B) MATERIALS AND METHODS

1. Animals

Male BALB/c (H-2^d) mice (University of Alberta, Edmonton, Alberta, Canada) were used as islet (aged 6-8 weeks) donors.

2. Cell Lines

L-cells (C3H mouse fibroblast cell line) were grown in DMEM (Gibco, Burlington, Ontario) supplemented with 10 % FBS, 2mM L-glutamine, and 100μg/ml P/S. L-cells that were deficient (MS) in the expression of the CI-MPR, also known as the M6P/IGFII receptor for granzyme B, or that were transfected to overexpress (MS9II) the CI-MPR were used as negative, and positive controls, respectively for receptor characterization and granzyme B uptake experiments. Jurkat cells (human T cell lymphoma line) were grown in RPMI 1640 (Gibco) supplemented with 10% FBS, 10^{-4} M

2-mercaptoethanol, 100μg/ml P/S, 20mM Hepes, and 80units/ml of human recombinant IL2 (RHFM). INS-1 cells (rat pancreatic β-cell line) were grown in RPMI Medium 1640 with L-glutamine and 11mM glucose (Gibco) supplemented with 10% FBS, 1% antibiotic/antimycotic, 2M sodium bicarbonate, 10mM Hepes, and 2-mercaptoethanol (17.7μl of a 1% solutions was added to 50mls of media, fresh each time) (11). InR1G9 cells (hamster pancreatic α-cell line) were grown in DMEM with 5mM glucose (Gibco) supplemented with 10% FBS, 1% antibiotic/antimycotic, and 3.7M sodium bicarbonate.

3. Isolation and Dissociation of Islets

Male BALB/c mice (aged 6-8 weeks) were used as islet donors. Access to the pancreas of these donors was gained through a midline incision. The pancreas was distended with collagenase (1mg/ml, Sigma Type V) in HBSS via the main pancreatic duct, excised, and placed in cold HBSS until all pancreases were harvested for digestion. Pancreases were then transferred to collagenase and digested in a shaking water bath for 13.45 minutes at 37°C. Following digestion with collagenase the tissue was given a quick hand shake to break up any chunks of tissue, and then cold HBSS was added to stop the digestion. Pancreatic tissue was pelleted, resuspended in fresh HBSS, and filtered through a 500μm mesh. Tissue was divided into tubes, pelleted, and Ficoll of varying densities (25%, 23%, 21.5%, and 11.5%; bottom to top respectively) was layered over the tissue in preparation for Ficoll density gradient centrifugation. Tissue was then spun at 2000 RPM for 10 minutes with no brake. Following this purification step islets were picked from the first layer of Ficoll and placed into a clean tube with HBSS and washed several times to remove the Ficoll. Islets were then put through a second Ficoll density gradient centrifugation, were picked again from the first layer, washed, and spun

at 2000 RPM for 2 minutes in preparation for dissociation into single cells. Islets were then resuspended in 40mls of calcium-free media containing 1mmol/l EGTA and 0.5% BSA (dissociation media) and transferred to a siliconized 250ml flask and agitated in a shaking water bath for 7 minutes at 37°C. Following this incubation, the flask was removed from the water bath and DNase (0.4mg/ml, Boehringer Mannheim, Laval, Canada) and trypsin (1mg/ml, Boehringer) were added to begin the digestion. The flask was returned to the shaking water bath for 5 minutes and monitored periodically by taking cell aliquots and checking the progress of the dissociation into single cells. Digestion was considered complete when 50% of cells were dissociated. At this time cells were transferred from the flask to a 50ml tube, spun at 1500 RPM for 5 minutes, and resuspended in HBSS. Cells were filtered through a 65μm filter and then used for the characterization of CI- and CD-MPR expression as well as granzyme B binding and uptake.

4. FACS Staining for CI- and CD-MPR

Dissociated mouse islets, INS-1, InR1G9, and control MS9II, MS, and Jurkat cells were added to 96-well plates at a concentration of 2×10^5 cells/well. Plates were spun down at 1500 RPM for 5 minutes. For CI-MPR and CD-MPR staining, dissociated islets, pancreatic cells lines, and control cell lines were incubated for 1 hour at 4°C with either 1) PBS with 0.1% BSA (50μl, control), or 2) rabbit anti-bovine CI-MPR (50μl, 1/500, William Brown, Cornell University), or 3) rabbit anti-human CD-MPR (50μl, 1/100, William Sly, Saint Louis University). Cells were washed with 180μl of PBS with 0.1% BSA and spun at 1500 RPM for 3 minutes. Cells were then incubated for 20 minutes at 4°C with goat anti-rabbit conjugated to Fluorescein Isothiocyanate (FITC,

1/100, Jackson, Mississauga, Ontario). Following this incubation, cells were washed with PBS supplemented with 2% FBS and fixed in PBS with 2% paraformaldehyde and 1% FBS (180µl) overnight at 4°C. Following the overnight fixation procedure, cells were washed several times with PBS/2% FBS prior to being resuspended in 180µl of PBS/2% FBS and transferred from the 96-well plate to 5ml falcon tubes for FACS (FACS scan, BD Biosciences) analysis of relative mean fluorescence intensity (MFI).

5. Binding and Uptake of Granzyme B

For the detection of granzyme B binding and uptake, target L-cells were incubated with fluorescein labeled granzyme B either at 4°C or 37°C for binding and uptake, respectively. At 4°C the CI-MPR remains arrested at the cell surface (also used for MPR staining) for the detection of granzyme B binding, whereas, at 37°C the CI-MPR gets internalized by the cell and granzyme B uptake can be measure. MFI of labeled cells is measured using flow cytometry with the MFI being proportional to the amount of granzyme B that is bound or taken up by the cell.

Dissociated mouse islets, INS-1, InR1G9, and control MS9II, MS, and Jurkat cells were added to 96-well plates at a concentration of 2×10^5 cells/well. Plates were spun down at 1500 RPM for 5 minutes. For granzyme B binding to dissociated islets, pancreatic cells lines, and control cell lines, cells were incubated for 1 hour at 4°C with either 1) PBS with 0.1% BSA (7µl, control), or 2) PBS with 0.1% BSA (2µl) and granzyme B conjugated to Alexa 488 (5µl, Molecular Probes). For granzyme B uptake into cells, cells were incubated for 1 hour at 37°C with either 1) DMEM with 0.1% BSA (25µl, control), or 2) DMEM with 0.1% BSA (20µl) and granzyme B conjugated to Alexa 488 (5µl, Molecular Probes). Following these incubations, cells were washed

with either PBS or DMEM with 0.1% BSA for 4°C and 37°C plates, respectively. Both plates were spun at 1500 RPM for 3 minutes and cells were fixed as described before and transferred to tubes for FACS analysis of MFI.

6. Statistical Analysis

CI- and CD-MPR expression as well as granzyme B binding and uptake data, in dissociated mouse islets and pancreatic cell lines, is presented as representative data from at least 3 different experiments.

V-C) RESULTS

1. CI- and CD-MPR Expression as well as Granzyme B Binding and Uptake in Dissociated Mouse Islets and Pancreatic Cell Lines

β-cells are highly auto-fluorescent because of their expression of flavin adenine dinucleotide (FAD) and can easily be distinguished from non-β-cells using FACS analysis (12). FACS analysis of dissociated islets showed two distinct populations upon examination of the FL-1 histogram (Figure 5-1). The population with higher fluorescence was considered to be the β-cell population (Figure 5-1A, B) and the other population was considered to be the non-β-cell population (Figure 5-1C, D).

Staining for the CI- and the CD-MPR revealed a high level of cell surface expression of both forms of the MPR on dissociated islets, as compared to controls (+ve control-CI-MFI=80, CD-MFI=15), with the CD-MPR (MFI=450) being predominant over the CI-MPR (MFI=90; Figure 5-2A). Furthermore, β-cells appeared to express higher levels of both forms of the MPR (CI-MFI=110, CD-MFI=700) than did non-β-cells (CI-MFI=20, CD-MFI=105). Granzyme B binding to cell surface MPR on dissociated islets (MFI=5) was comparable to positive control levels (MFI=10); however,

granzyme B uptake (MFI=10) was poor as compared to controls (MFI=78; Figure 5-2B). Granzyme B binding and uptake was slightly higher in β-cells (binding-MFI=5, uptake-MFI=10) as compared to non-β-cells (binding-MFI=1, uptake-MFI=4).

Staining for the CI- and the CD-MPR in pancreatic cell lines revealed a moderate amount of expression of both forms of the MPR, as compared to controls (CI-MFI=79, CD-MFI=150) with the levels of CI- (MFI=25) and CD- (MFI=25) MPR being relatively equal in both INS-1 and InR1G9 cell lines (Figure 5-3A). Granzyme B binding (MFI=4) and uptake (MFI=19-27) by pancreatic cell lines was relatively good, although less than in controls (binding-MFI=11, uptake-MFI=50; Figure 5-3B).

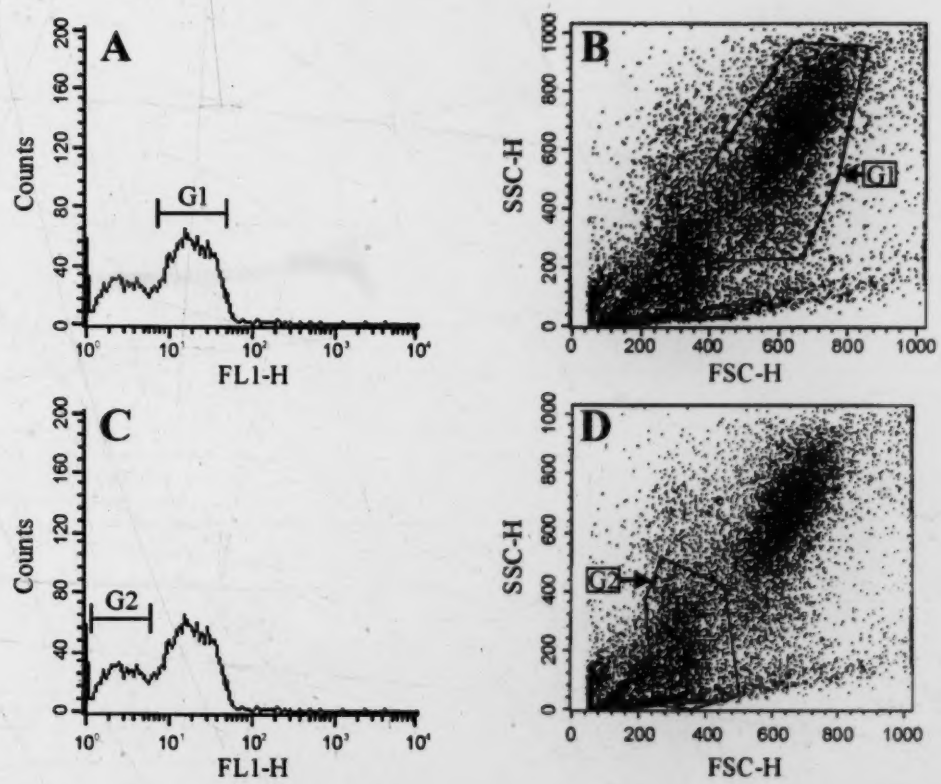
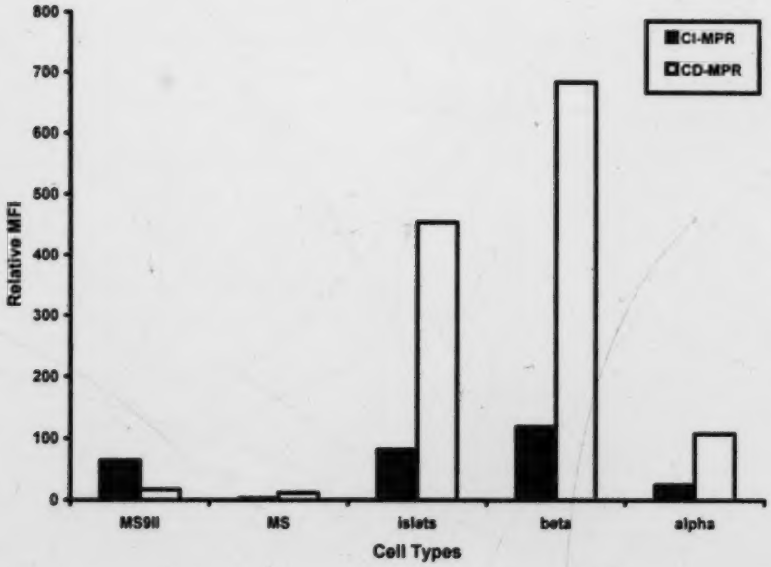


Figure 5-1. FACS identification of β- and non-β-cells in a sample consisting of dissociated islets. β-cells can be distinguished from non-β-cells due to their high level of endogenous auto-fluorescence. (A) FL-1 histogram with gate (G1) on β-cell population. (B) Dot plot with gate (G1) on β-cell population determined from FL-1 histogram. (C) FL-1 histogram with gate (G2) on non-β-cell population. (D) Dot plot with gate (G2) on non-β-cell population determined from FL-1 histogram. SSC stands for side scatter and represents the granularity of the cells. FSC stands for forward scatter and represents the size of the cells. FL-1 stands for level of fluorescence.

A



B

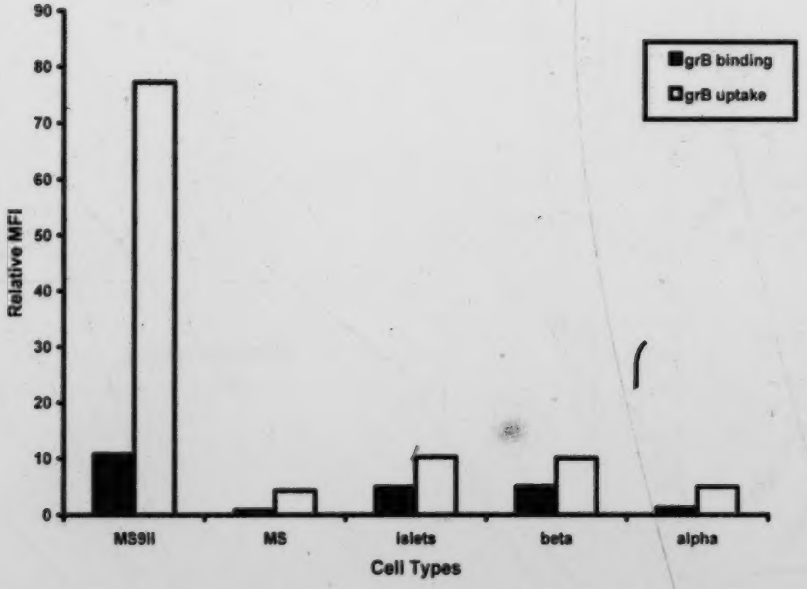
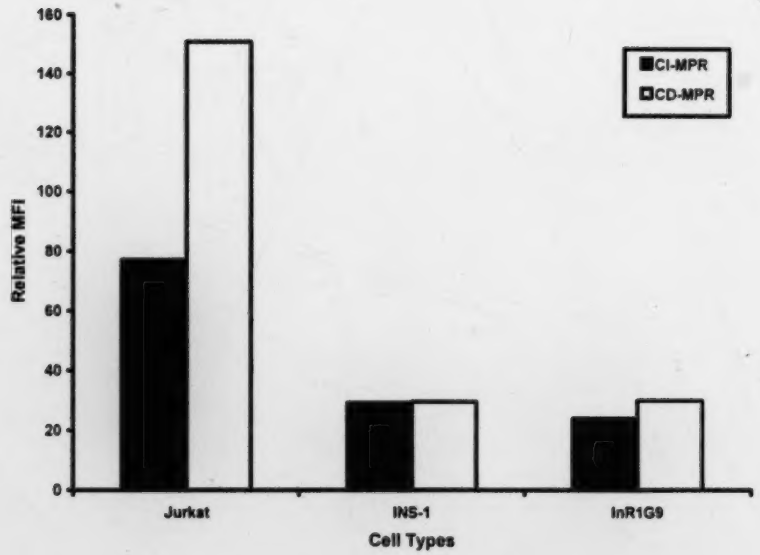


Figure 5-2. (A) Cation-independent and -dependent (CI/CD) forms of the mannose-6 phosphate receptor (MPR) for granzyme B in dissociated mouse islets. (B) Granzyme B (grB) binding and uptake in dissociated mouse islets. MS9II and MS fibroblast L-cells were used as positive and negative controls for MPR expression as well as granzyme B binding and uptake, respectively. Data shown are representative of three independent experiments. MFI-mean fluorescence intensity.

A



B

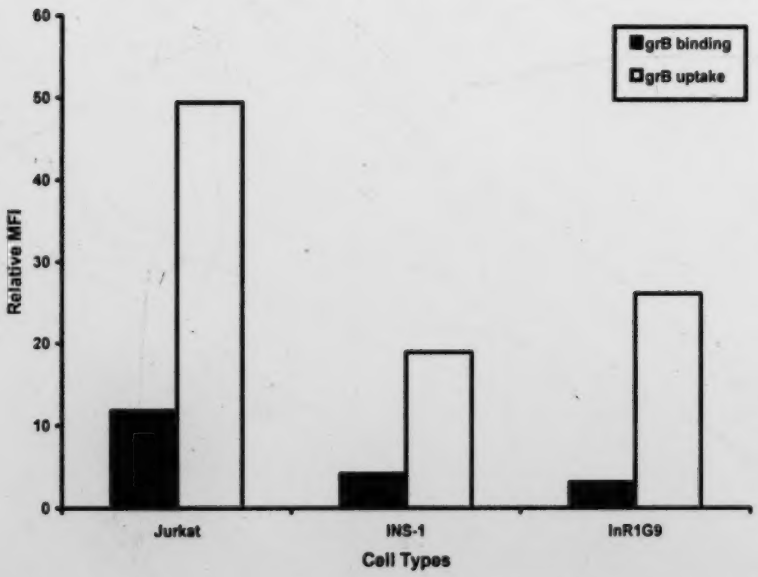


Figure 5-3. (A) Cation-independent and dependent (CI/CD) forms of the mannose-6 phosphate receptor (MPR) for granzyme B in pancreatic cell lines (rat INS-1 β-cell line, and hamster InR1G9 α-cell line). (B) Granzyme B (grB) binding and uptake in pancreatic cell lines. Human Jurkat cells were used as a positive control for MPR expression as well as granzyme B binding and uptake, respectively. Data shown are representative of three independent experiments. MFI-mean fluorescence intensity.

V-D) DISCUSSION

In the present study we found that dissociated islets expressed very high cell surface levels of both the CI- and CD-MPR; however, despite high MPR cell surface expression dissociated islets showed very poor uptake of granzyme B. This phenomenon could be explained by the extremely high cell surface expression of the CD-MPR observed in dissociated islets, with the CD-MPR being predominant over the CI-MPR. The CI-MPR has been found to be the principle form of the MPR responsible for granzyme B uptake (6) and it may be that very high levels of CD-MPR cell surface expression interferes with granzyme B uptake. This could happen if the CD-MPR is acting like a sink to bind granzyme B but ineffectively internalize it. Additionally, we saw higher MPR expression as well as granzyme B binding and uptake in β-cells versus non-β-cells. The interpretation of this finding is unclear but this may explain in part why β-cells are specifically targeted for destruction in type 1 diabetes.

Subsequently, we tested two pancreatic cell lines, rat INS-1 β-cell line and hamster InR1G9 α-cell line, for CI- and CD-MPR expression as well as granzyme B binding and uptake. We found that CI- and CD-MPR cell surface expression was relatively the same in both INS-1 and InR1G9 cells and that granzyme B binding and uptake was much improved as compared to dissociated islets. This may be because CD-MPR cell surface expression is much lower in these pancreatic cell lines.

We found that it was much easier to use and the results were much better for pancreatic cell lines as compared to dissociated islets for the examination of the granzyme B-MPR pathway. No extensive isolation or dissociation process was required for pancreatic cell lines that were easily maintained in tissue culture. Additionally,

granzyme B uptake in the cell lines was much improved as compared to the dissociated islets. Initial studies of the granzyme B-MPR pathway can be easily studied in pancreatic cell lines and then proven later in primary islets.

In conclusion, we found that granzyme B does get bound and internalized by cell surface CI- and CD-MPR in dissociated islets and pancreatic cell lines. However, pancreatic cell lines were much easier to work with and granzyme B uptake was more significant as compared to dissociated islets. Development of techniques for studying the granzyme B-MPR pathway to cell death in islets will allow the future testing of new therapeutics aimed at blocking this pathway that may be able to prevent graft rejection.

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CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION

VI-A) GENERAL DISCUSSION

Type 1 diabetes, characterized by elevated blood glucose, results from the autoimmune destruction of the insulin secreting β-cells located in the islets of Langerhans of the pancreas (1). Insulin injection, blood glucose monitoring, a healthy diet, and exercise are all required for the maintenance of blood glucose and the management of type 1 diabetes (2). However, even today, with advances in insulin formulations and administration, micro- and macrovascular complications, such as neuropathy, nephropathy, retinopathy, and cardiovascular disease, are common causes of morbidity and mortality in patients with type 1 diabetes (3-5). The Diabetes Control and Complications Trial (DCCT) demonstrated that tight blood glucose control, achieved through multiple daily insulin injection or pump therapy, could prevent microvascular complications of diabetes (6). However, the DCCT also found an increase in the frequency of episodes of low blood glucose (hypoglycemia) with intensive insulin therapy. Furthermore, tight blood glucose control is rarely achieved by the average person with diabetes making complications more likely (7).

Islet transplantation using what has come to be known as the Edmonton protocol has been tremendously successful at helping dramatically improve blood glucose control and quality of life in many patients with type 1 diabetes (8-11). However, despite recent advances, waiting lists for this procedure are very long and even very brittle diabetics

whose life depends on this procedure have to wait due to the shortage of human pancreases from which islets can be procured for transplantation. Secondly, the ultimate goal of islet transplantation is to be able to offer the procedure to young, even newly diagnosed, type 1 diabetic patients in order to restore their blood glucose control early enough to prevent the devastating complications of diabetes. However, currently the risks associated with a life-time of immunosuppression outweigh the benefits of the procedure. Therefore, the goal of current research is finding an unlimited supply of tissue for islet transplantation that can be transplanted in such a way that chronic immunosuppressive therapy is not required. Even though this appears to be a lofty goal, numerous avenues are being explored to achieve it.

Many sources of tissue are currently being considered for islet transplantation including: living islet donors, beta cell lines, stem cells (both embryonic and adult), neonatal porcine islets, and fish Brockmann bodies (12-16). Another alternative is to engineer non-beta cells (eg. intestinal K-cells, or hepatocytes), within the person who has diabetes, to secrete insulin (17;18). However, all of these options would still require immunosuppressive therapy to prevent graft rejection and autoimmune disease recurrence.

Several strategies exist for the protection of islet grafts from immune destruction including: encapsulation, co-stimulation blockade, mixed chimersim, dendritic cell therapy, intrathymic administration of donor antigen, and transplantation into immune privileged sites or co-transplantation with immune privileged tissue (11;19-21). It is likely that a combination of these strategies will be the most effective at preventing graft rejection.

We focused on the use of Sertoli cells, an immune privileged tissue shown to protect islets from auto-, allo-, and even xenoimmune mechanisms of destruction without the requirement for chronic immunosuppressive therapy (21-27). More specifically, we focused on optimizing islet/Sertoli cell co-transplantation conditions in an allogeneic mouse model of chemically induced diabetes. We additionally focused on uncovering the mechanism of how Sertoli cells protect themselves and bystander cells from immune destruction by analyzing the effect of products secreted by Sertoli cells on various aspects of immune function.

It has been shown previously that co-transplantation of islets with an optimal amount of Sertoli cells can prolong islet allograft survival in rodent models of chemically induced diabetes; however, too few or too many Sertoli cells resulted in shorter allograft survival times (23-25;28). What had not been addressed previously was the reason why Sertoli cells were able to prolong islet allograft survival in some cases and not in others. To address this question we co-transplanted 500 BALB/c islets with 1, 2, 4, or 8 x 10^6 BALB/c Sertoli cells beneath the kidney capsule of streptozotocin induced diabetic BALB/c, C3H, and C57BL/6 mice and subsequently characterized both successful and unsuccessful islet/Sertoli cell grafts using immunohistochemistry.

We found that co-transplantation of islets with Sertoli cells in a syngeneic mouse model had no deleterious effect on islet graft function which seems to be consistent with other findings in the BALB/c mouse (28). Extension of islet allograft survival with Sertoli cells, in our study, was found to depend on the amount of Sertoli cells transplanted and the strain of the recipient mouse. Co-transplantation of islets with 4 x 10^6 Sertoli cells was found to be the optimal amount of Sertoli cells for extended islet

allograft survival in the C3H mouse. Co-transplantation with fewer (1 or 2 x 10^6) or more (8 x 10^6) Sertoli cells resulted in shorter islet allograft survival time. However, in the B6 mouse co-transplantation of islets with 1 or 2 x 10^6 Sertoli cells was found to be the optimal amount of Sertoli cells for extended islet allograft survival, whereas, co-transplantation with more (4 or 8 x 10^6) Sertoli cells resulted in shorter islet allograft survival time. Additionally, fewer islet allografts survived beyond 60 days in the B6 mouse. These findings suggest that fewer Sertoli cells are required for extended islet allograft survival in the B6 mouse as compared to the C3H mouse; however, 8 x 10^6 Sertoli cells was consistently too many. Furthermore, Sertoli cells appeared to prolong islet allograft survival more effectively in the C3H mouse as compared to the B6 mouse, even at optimal amounts of Sertoli cells.

Subsequently, we wanted to examine successful and unsuccessful islet/Sertoli cell grafts to try and understand the Sertoli dose and recipient strain effects. Examination of successful islet/Sertoli cell grafts, harvested from normoglycemic mice at day 80 post-transplantation, revealed Sertoli cells in tubule-like structures, similar to how they are arranged in their native testis. Conversely, unsuccessful islet/Sertoli cell grafts, harvested from diabetic mice at the time of rejection revealed Sertoli cells arranged randomly or in aggregates. In both cases islets appeared fragmented and surrounded the Sertoli cell structures. This may explain why successful Sertoli mediated protection of islet allografts was found to be dependent on both Sertoli number and recipient mouse strain. For example, too few Sertoli cells may not be enough for tubule formation and too many may lead to hypoxic and nutrient-deprived conditions resulting in necrosis and inflammation and subsequent loss of Sertoli cell mass, also resulting in poor tubule

formation. Furthermore, strong immune responses to allografts may also prevent Sertoli tubule formation, as we found to be the case in the B6 mouse.

We also examined CD4 and CD8 T-cell as well as macrophage infiltrate in successful and unsuccessful islet/Sertoli cell grafts. Unsuccessful grafts had marked CD4 and CD8 T-cell as well as macrophage infiltrate. Successful grafts had practically no CD8 T-cell infiltrate but still had some CD4 T-cells and macrophages. These findings suggest that Sertoli cells may be effective at protecting islet allografts from CD8 T-cells but CD4 T-cells and macrophages may still result in future graft destruction that is not prevented by Sertoli cells.

Subsequently, we prepared Sertoli cell conditioned media (SCCM) from cultured Sertoli cells isolated from the testis of 9-12 day old BALB/c mice and tested the ability of the SCCM to inhibit immune effector mechanisms. Rat SCCM has previously been found to inhibit mitogen stimulated lymphocyte proliferation (29-31). To test the biological activity of our mouse SCCM we examined the effect of our preparation on concanavalin-A (Con-A) stimulated lymphocyte proliferation. We found that our SCCM was indeed biologically active with inhibitory effects seen on Con-A stimulated lymphocyte proliferation similar to what has been described in the past.

Since Sertoli cells were found to effectively inhibit CD8 T-cell infiltrate in our studies it appeared that Sertoli cells may have some sort of effect on this T-cell population. The CD8 positive cytotoxic T lymphocyte (CTL) is one of the main immune mediators in graft rejection (32-35). We tested our SCCM for its effect on CTL and granzyme B mediated killing of target L-cells. Granzyme B is the main CTL component involved in inducing DNA fragmentation and apoptotic cell death in CTL target cells

(36). We found that SCCM effectively inhibited both CTL and granzyme B mediated apoptosis.

Next, we were interested in examining how SCCM affects the biological activity of granzyme B. When granzyme B is released from the CTL it enters the target cell via its receptor, the mannose-6 phosphate/insulin-like growth factor-II (M6P/IGF-II) receptor (37). Granzyme B is subsequently released from the endocytic vesicle into the cytoplasm by perforin (38). Once in the cytoplasm, granzyme B, a serine proteinase, cleaves pro-caspases at aspartic acid residues, activating them and initiating the caspase cascade to apoptotic cell death (39-42). Sertoli cells have been found to secrete multiple factors that may interfere with the granzyme B pathway to cell death. Sertoli cells secrete multiple ligands for the M6P/IGF-II receptor including 10 different mannose-6 phosphorylated (M6P) glycoproteins and IGF-II (43;44). Sertoli cells have also been found to express human proteinase inhibitor-9 (PI-9), a potent inhibitor of granzyme B enzymatic activity (45;46). We found that SCCM did not significantly reduce M6P/IGF-II receptor expression, nor did it significantly reduce granzyme B binding or uptake by target cells. However, we did find that SCCM effectively inhibited human and mouse granzyme B enzymatic activity which may explain our observed effects of SCCM on granzyme B biological activity.

Finally, we wanted to identify the factor secreted by Sertoli cells that was affecting granzyme B enzymatic and biological activity. We found that the factor in SCCM formed an SDS stable complex with granzyme B. Serpins are known to inhibit serine proteinases through the formation of essentially irreversible complexes (47). The mouse serpin responsible for the inhibition of granzyme B is serine proteinase inhibitor-6

(SPI-6) (48). We subsequently prepared samples of SCCM with granzyme B for SDS-PAGE and Western blot analysis. Staining for SPI-6 and granzyme B revealed the presence of a higher molecular weight complex in the lane with SCCM and granzyme B that was positive for granzyme B but not SPI-6 as anticipated. These findings indicate that some yet unidentified factor secreted by Sertoli cells, probably a serpin, interacts with granzyme B to form a stable complex that interferes with granzyme B enzymatic and biological activity.

VI-B) CONCLUSION

In conclusion, our data show that Sertoli cells may be used as a less-toxic alternative to chronic immunosuppressive therapy in islet transplantation for the prevention of islet allograft rejection. We found, as have others, that Sertoli cell number co-transplanted with islets was critical for extended islet allograft survival in the mouse model of chemically induced diabetes. Secondly, successful prolongation of islet allograft survival with Sertoli cells appeared to also depend on recipient mouse strain. Our observation that Sertoli cells formed tubule-like structures in successful islet/Sertoli cell grafts may explain why Sertoli cell number and recipient mouse strain are important. Perhaps tubule formation is dependent on an optimal number of Sertoli cells, the number of which may vary from mouse strain to mouse strain. Additionally, the strength of the recipient immune response may also dictate Sertoli cell tubule formation. We also found that Sertoli cells were successful at preventing CD8 T-cell but not CD4 T-cell and macrophage infiltrate which may interfere with prolonged graft acceptance. Closer examination of the effect of SCCM on CD8 T-cell effector mechanisms revealed that a

factor secreted by Sertoli cells effectively inhibited granzyme B enzymatic and biological activity through the formation of a stable complex. Our experiments show that this factor is not SPI-6 but perhaps some other, yet unidentified, serpin.

It is clear that under certain conditions Sertoli cells can protect islet allografts from CD8 CTL killing; however, additional immunosuppressive therapies may be required for protection from CD4 T-cell and macrophage killing in order to achieve long-term graft acceptance. The importance of Sertoli cell tubule formation post-cotransplantation with islets is unknown but it may be that this helps Sertoli cells focus secreted immunosuppressive factors in the direction of the islet allograft for maximum protection from immune destruction. Clearly, more work needs to be done to further understand what is required for optimizing islet/Sertoli cell co-transplantation for prolonged islet allograft survival. However, these initial experiments in the mouse model will help pave the way for future work involving the optimizing of islet/Sertoli cell co-transplantation conditions in the neonatal pig model to potentially overcome both the tissue supply and chronic immunosuppression challenges that face islet transplantation.

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APPENDIX A

MOUSE SERTOLI CELL CONDITIONED MEDIA DOES NOT PROLONG ISLET ALLOGRAFT SURVIVAL IN STREPTOZOTOCIN INDUCED DIABETIC MICE

A-1) OBJECTIVE

To determine whether or not mouse Sertoli cell conditioned media (SCCM) can prolong islet allograft survival in an *in vivo* mouse model of streptozotocin induced diabetes.

A-2) RATIONALE

Testicular Sertoli cells have been shown to prolong islet allograft survival in rodent models of chemically induced diabetes (1-4). Sertoli cells are known to secrete many proteins involved in active immune suppression (5-10). Through the course of this thesis we have discovered that Sertoli cell conditioned media (SCCM) has the ability to suppress immune effector functions *in vitro*, including lymphocyte proliferation, and CTL granzyme B-mediated apoptosis (Chapters III and IV). The present study was designed to assess the ability of SCCM to prolong islet allograft survival in an *in vivo* mouse model of diabetes.

A-3) EXPERIMENTAL PROTOCOL

BALB/c mouse Sertoli cells were isolated, cultured, and the supernatant collected, and concentrated for the preparation of SCCM as described previously (Chapter IV). BALB/c mouse islets were isolated, cultured overnight, and aliquots of 500 each were transplanted beneath the left kidney capsule of streptozotocin-induced diabetic B6 mice as described previously (Chapter II). B6 islet transplant recipients were given 300μl of SCCM intraperitoneally once daily for the first 15 days post-transplantation (starting on the day of the transplant). Control B6 mice received an islet transplant but with no injection treatment. Recipients were allowed to recover and blood glucose was monitored three times a week until the time of rejection (return to hyperglycemia; two consecutive readings of ≥ 18mmol/l) when the graft bearing kidneys were harvested for immunohistochemical analysis (Chapter II). Values shown are the average ± the standard error of the mean (SEM).

A-4) RESULTS

We found that BALB/c islets were rejected by B6 mouse recipients on average at 25.8 ± 5.5 days post-transplantation (Table A-1). This was significantly longer than in our previous group where we found BALB/c islet graft survival in B6 mice to be on average approximately 13.8 ± 1.1 days (Chapter II). Treatment of recipient B6 mice with SCCM did not significantly prolong BALB/c islet allograft survival. Instead, graft survival was actually decreased to an average of 20.4 ± 1.6 days. Co-transplantation of BALB/c islets with 1 or 2 x 10^6 BALB/c Sertoli cells in B6 mice did, in comparison, significantly prolong islet allograft survival (Chapter II). However, in general BALB/c

islet grafts fared better in C3H recipients with and without Sertoli cells than in B6 recipients.

Table A-1. Survival of 500 BALB/c islets transplanted beneath the kidney capsule of diabetic B6 recipients treated with either no Sertoli cell conditioned media (SCCM) or a once daily injection of 300µl of SCCM.

SCCM Dose (µl)	Day of Graft Rejection	Graft Survival Time (Days)
0	13,17,20,21,35,>49	>25.8 ± 5.5
300	15,16,18,19,19,23,24,29	20.4 ± 1.6

Graft survival expressed as the mean ± SEM. >Graft-bearing kidneys were harvested from normoglycemic animals on days indicated to confirm return to hyperglycemia and for immunohistochemical analysis.

A-5) CONCLUSION

It is our conclusion that Sertoli cells themselves need to be present in the islet graft for optimal protection from immune destruction. Sertoli cell conditioned media alone was not effective at preventing islet allograft destruction *in vivo* despite our findings *in vitro* that it can inhibit immune responses. This may simply be a matter of not having the Sertoli cell conditioned media concentrated enough, in the localized environment of the graft for optimal protection. Continuous infusion of highly concentrated Sertoli cell conditioned media in the localized environment of the graft achieved through an infusion set may mimic the Sertoli co-transplant setting. However, the optimal scenario is still the co-transplantation of islets with Sertoli cells for localized secretion of immunosuppressive factors resulting in prolonged graft survival.

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APPENDIX B

LONGTERM SURVIVAL OF MOUSE ISLET ALLOGRAFTS IN THE ABSENCE OF IMMUNOSUPPRESSION

B-1) OBJECTIVE

To establish a mouse model for co-transplantation of islets with Sertoli cells for optimization of conditions required for Sertoli cells to prolong islet allograft survival.

B-2) RATIONALE

Prior to starting co-transplantation of islets with Sertoli cells (Chapter II) we wanted to find the best strain combination that would give a strong immune response to islet allografts transplanted alone to be able to see an effect of Sertoli cells in prolonging islet allograft survival. A further limitation of our study was that we wanted both islets and Sertoli cells to come from the same mouse strain, for a purely allogeneic combination, and as Sertoli cells were easily obtained locally from BALB/c pups (9-12 days, University of Alberta animal facility), we wanted to obtain islets from local BALB/c mice as well. Unexpectedly, we discovered that the first MHC-mismatched pair that we chose [BALB/c islets ($H2^d$) into diabetic CBA/J recipients ($H2^k$)] resulted in longterm acceptance of islet allografts without any form of immunosuppressive therapy. The results are presented and discussed herein.

B-3) EXPERIMENTAL PROTOCOL

BALB/c or CBA/J mouse islets were isolated, cultured overnight, and aliquots of 500 each were transplanted beneath the left kidney capsule of streptozotocin-induced diabetic CBA/J or BALB/c mice, respectively as described previously (Chapter II). CBA/J mouse recipients were rendered diabetic with a streptozotocin (Sigma, Canada, Oakville, Ontario) dose of 375mg/Kg (intravenously) 2-3 days before transplantation. Recipients were allowed to recover and blood glucose was monitored three times a week until the time of rejection (return to hyperglycemia; two consecutive readings of ≥ 18mmol/l) when the graft bearing kidneys were harvested for immunohistochemical analysis (Chapter II). Values shown are the average ± the standard error of the mean (SEM).

B-4) RESULTS

We found that when BALB/c islets were transplanted into CBA/J recipients, islet allograft survival was >65.3 ± 3.9 days (Table B-1). Insulin and hematoxylin/eosin (H/E) staining of BALB/c islet grafts harvested from euglycemic CBA/J recipients revealed the presence of intact, insulin positive islets with peripheral islet cellular infiltrate (Figure B-1). However, when we reversed this combination and transplanted CBA/J islets into BALB/c recipients, we found that islet allografts were rejected by day 25.9 ± 2.2 with no grafts surviving beyond 60 days. Insulin and H/E staining in this case revealed very little insulin positive cells, with massive cellular infiltrate (not shown).

Table B-1. Survival of 500 BALB/c islets transplanted beneath the kidney capsule of diabetic CBA/J recipients and survival of 500 CBA/J islets transplanted beneath the kidney capsule of diabetic BALB/c recipients.

Group	Day of Graft Rejection	Graft Survival Time (Days)
BALB/c islets into CBA/J	42,49,>69,>69,>69,>69,>69,>76,>76	>65.3 ± 3.9
CBA/J islets into BALB/c	18,20,20,20,21,21,22,26,26,31,33,33,46	25.9 ± 2.2

Graft survival expressed as the mean ± SEM. >Graft-bearing kidneys were harvested from normoglycemic animals on days indicated to confirm return to hyperglycemia and for immunohistochemical analysis.

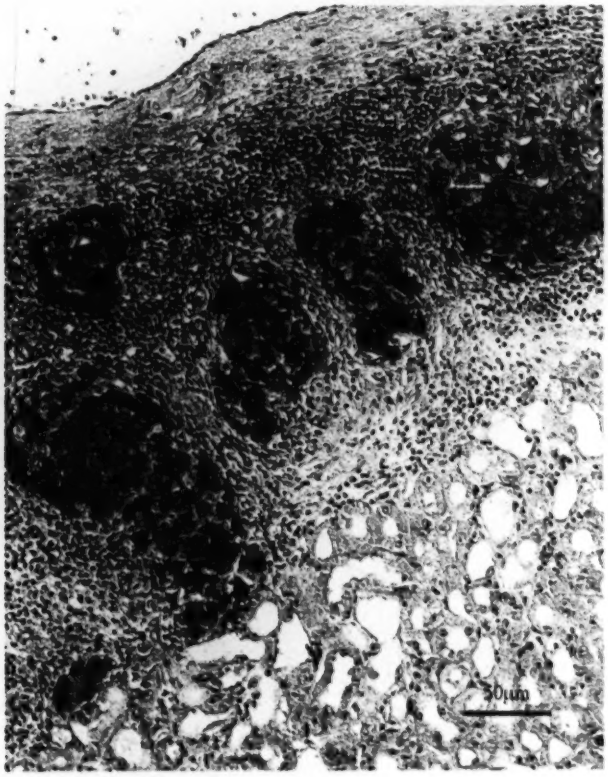

Figure B-1. Insulin and hematoxylin/eosin staining of an accepted BALB/c islet allograft (500 islets) harvested from a euglycemic CBA/J recipient on day 30 post-transplantation. Intact islets are seen with peripheral islet cellular infiltrate.

B-5) CONCLUSION

We found, unexpectedly, that BALB/c islet allografts survived for extended periods of time in diabetic CBA/J recipients. However, reversal of this strain combination, or the transplantation of CBA/J islet allografts into diabetic BALB/c recipients led to timely graft destruction. This suggests that the CBA/J and not the BALB/c mouse must be immune compromised in some manner. Previous studies have suggested that either diabetes or streptozotocin can be immunosuppressive (1;2). Furthermore, the transplantation of clean (hand-picked islets) and tissue culture have been shown to improve islet allograft survival (3;4). Perhaps a combination of factors is at work in our situation culminating in prolongation of islet allograft survival in the CBA/J mouse. However, these findings illustrate the importance of finding an appropriate mouse model for islet co-transplantation with Sertoli cells in order to see true prolongation of islet allograft survival.

B-6) REFERENCES

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